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TOXINS AND TOXICITY OF PROTOGONYAULAX FROM THE NORTHEAST PACIFIC

University of Alaska PH.D. 1982

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TOXINS AND TOXICITY OF *PROTOGONYAULAX* FROM THE NORTHEAST PACIFIC

A

THESIS

Presented to the Faculty of the University of Alaska

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Sherwood Hall, M.S.

Fairbanks, Alaska

December 1982

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TOXINS AND TOXICITY OF *PROTOGONYAULAX*

FROM THE NORTHEAST PACIFIC

RECOMMENDED:

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Program Head

Director, Division of Marine Sciences

APPROVED:

Vice Chancellor for Research and Advanced Study

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ABSTRACT

Dinoflagellates of the genus *Protogonyaulax* contain a group of substances that can be lethal to many creatures, including man, and may accumulate at many points in the food web. The substances are most familiar as paralytic shellfish poison (PSP), which occurs sporadically in bivalves. The present study was undertaken because previous work left in doubt both the origin and chemical nature of the toxins along the Alaskan coast.

To investigate the problem, dinoflagellates were isolated from locations along the Pacific coast ranging from San Francisco to Dutch Harbor. Most isolates were obtained by incubating subtidal sediments to germinate resting cysts. Toxic isolates were obtained from most locations sampled. On the basis of morphology, all toxic isolates fell within the genus *Protogonyaulax*. The growth and toxicity of one clone (PI07) was studied under a variety of culture conditions. Toxicity was greatly suppressed under the conditions traditionally employed for culturing *Protogonyaulax*, suggesting that the toxicity of cells in nature may in general be higher than has been recognized.

Chemical studies of the toxins extracted from *Protogonyaulax* revealed that the six toxins previously known (saxitoxin, its N-1-hydroxyl and 11-hydroxysulfate derivatives) are generally accompanied by somewhat larger amounts of their 21-sulfo derivatives. These have likely not been recognized in past studies due to their greatly reduced toxicity, facile hydrolysis, and altered chromatographic properties.

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The toxin composition of several isolates was determined and indicates that toxin composition is a conservative property of each clone and that there are regional populations of *Protogonyaulax* with uniform toxin composition, but that toxin composition differs substantially among regions. This pattern of variation, coupled with the great differences in the properties of the toxins, indicates that the nature of PSP will similarly vary from one region to another but will be uniform within each.

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ACKNOWLEDGEMENTS

Funding for this work was provided by a Graduate Fellowship in Resource Problems from the University of Alaska, a Graduate Fellowship from the Alaska Sea Grant Program, grants from the United States Food and Drug Administration, the National Marine Fisheries Service, and the Alaska Sea Grant Program, and direct appropriations from the State of Alaska and the Institute of Marine Science, University of Alaska. I would like to express my appreciation to these agencies for their support, and particularly to Dr. E. P. Ragelis for his efforts to encourage federal support of studies on toxins in marine food products.

The successful outcome of the work was greatly aided by the excellent facilities provided by the Institute of Marine Science at the Seward Marine Center.

I would like to thank the members of my committee for their helpfulness, accessibility and encouragement.

I would like to express my appreciation to Dr. H. K. Schnoes for offering the use of his laboratory at the University of Wisconsin, and to his students Dr. C. Fix Wichmann and Mr. F. E. Koehn for their assistance; to Dr. S. D. Darling for his tireless labors in solving the x-ray structures; to Mr. G. Mueller and Dr. R. Horner for their encouragement, many helpful discussions, and for taking the photomicrographs; to Ms. L. Nishitani for providing the starter cultures used in the preliminary phase of this work, and for many helpful discussions; to Dr. F. J. R. Taylor for comments on taxonomy; to Dr. R. C. Fay for early guidance and continued encouragement; to the staff of the Institute of Marine Science,

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both in Fairbanks and at the Seward Marine Center for their able and willing assistance, often under stress; and to my family and friends for their support and encouragement.

I would particularly like to thank Dr. G. L. Boyer for his helpfulness and willingness to openly discuss his work, providing the benchmark that allowed me to get my bearings in dealing with the toxins.

For their help in the final production of this thesis, I would like to thank Helen Stockholm and her assistants Mauricette Nicpon and Ruth Hand for their energetic and skillful typing of the manuscript, and Phyllis Shoemaker for printing the photographs.

Finally, I would like to express my appreciation to my major professor, Dr. Paul B. Reichardt, but it is difficult to find the appropriate phrases. He has provided calm and patient guidance throughout the course of this study. I could say that I learned a lot from him, or that I followed the example he set, but both would be a bit presumptuous. The opportunity was there. The example he has provided, both as a human being and as a professional, has been an inspiration. He sets high standards not by insisting on them, but by his own performance. I have been very fortunate to have him as a teacher.

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I. INTRODUCTION

I.A. Perspective

Paralytic shellfish poisoning (PSP) is most familiar as toxicity to humans from bivalves that, unaffected by the toxins, consume and accumulate toxin from dinoflagellates. However, bivalves are not the sole accumulators of the toxins, nor uniformly insensitive, and humans are not the only victims. Broadly considered, the situation instead appears to be one of the toxins moving through the food chain, various organisms differing in their susceptibility to the toxins and tendency to accumulate them. The term toxin is in itself subjective, there being many organisms to which these substances clearly are not toxic and the toxicity of the substances providing no apparent benefit to the organisms that appear to produce them. As with fire and other tools, the toxins pose a threat to man largely because of the primitive state of our understanding of their nature and occurrence. While minimizing the negative impacts of the toxins will likely remain a primary concern, with proper understanding we can turn their unique properties to good use. Already the toxins are vital tools in neurophysiological studies because of the specific mechanism by which they act, and they may provide the basis for developing a novel class of local anaesthetics.

The work described in part II of this dissertation involves the study of dinoflagellates belonging to the genus *Protogonyaulax*, the toxins they contain, and how that content varies. In part IV, the results will be discussed by viewing the toxins from three perspectives:

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their relationship to *Protogonyaulax*, their selective binding properties, and their movement through the food chain.

Despite broader implications, study in this area has been prompted, or justified, largely by concern over shellfish toxicity. Understandably, the most dramatic progress has occurred following major outbreaks, the most notable being those in Wilhelmshaven, around 1885 (19 victims, 4 deaths; Virchow, 1885), in San Francisco in 1927 (102 victims, 6 deaths; Meyer *et al.*, 1928), and in New England in 1972 (33 victims, no deaths; Anon., 1972). From these numbers it is apparent that the toll, compared to other hazards, is relatively small.

Modern understanding of the problem stems largely from work under the leadership of Karl Meyer and Hermann Sommer at the University of California, San Francisco, through the decade following the 1927 episode. The problem has been extensively reviewed (McFarren *et al.*, 1961; Halstead, 1965; Quayle, 1969; Prakash *et al.*, 1971), and was a major subject of two recent symposia (LoCicero, 1975; Taylor and Seliger, 1979). Developments in toxin chemistry have been reviewed by Schantz (1960; 1971; 1979) and Shimizu (1978; 1979).

I.B. Pharmacology

Symptoms of PSP appear quickly, within minutes to hours, and are principally neurological. In severe cases, death may result from suffocation due to respiratory paralysis (Permewan, 1888; Evans, 1965). Since the toxins leave the system quickly, resuscitation is practical and generally assures recovery of the patient. The toxins do have a

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relatively weak hypotensive effect which may supervene as a result of extremely large doses (Prinzmetal *et al.*, 1932; Evans, 1965; Kao, 1975). However, only three cases have been recorded where the victim died due to vascular collapse despite respiratory support (Permewan, 1888; Sapieka, 1958; Sharpe, 1981). Survivors recover promptly, within a period of days, the toxins appearing to have no lasting effects.

Apparent potency of the toxins varies widely, depending on the recipient and the circumstances of administration. From epidemiological surveys, such as Medcoff *et al.* (1947), it appears that human sensitivity varies, there being some evidence suggesting that people who normally consume shellfish are less susceptible. Symptoms following ingestion are generally intensified by alcohol or other substances that accelerate absorption in the gastrointestinal tract or increase peripheral circulation, or when shellfish are consumed on an empty stomach.

From experiments on mice and rabbits, shellfish poison appears to be about 40 times more potent when administered by intraperitoneal injection than when given orally (Prinzmetal *et al.*, 1932). Injection into rabbit and mouse ileum suggested that the difference is due to reduced adsorption through the gastrointestinal tract, rather than destruction of the toxin.

As will be discussed in section IV.B.2b, the transmission of impulses along nerve and muscle membranes depends in part on a transient increase in permeability to sodium ions, presently modelled as the brief opening of a 'sodium channel'. The toxins act by a specific, reversible blockage of this transient increase in conductance.

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I.C. Occurrence

PSP is predominantly associated with, but by no means restricted to, bivalves in the summer in the temperate zone of the northern hemisphere. Such toxicity has been known to occur throughout the year, in both hemispheres, in the tropics, and in animals other than bivalves. Only a small part of the total resource is toxic, the magnitude of the problem being determined by threat and uncertainty since toxicity is sporadic in its occurrence and difficult to monitor.

I.D. Cause

The most dramatic step in the understanding of PSP was the demonstration by the Sommer and Meyer group (Sommer *et al.*, 1973) that toxicity in California mussels resulted from the consumption and accumulation of toxins from the dinoflagellate *Gonyaulax catenella*, first recognized as a new species (Whedon and Kofoid, 1936) in the course of their studies. That the toxin was accumulated from a food organism seems obvious in retrospect, but it had been an obvious possibility for over a century before Sommer and his associates, through perseverance and some good luck, managed to find proof. Combe (1828) had considered food or other water-born sources in an episode (about 30 victims, two deaths) involving the consumption of mussels in Lieth, Scotland (near Edinburgh). Unfortunately, the proximity of toxic to apparently non-toxic mussels led him to discard this possibility. Following the Wilhelmshaven outbreak in 1885, Schmidtmann (1888) found that non-toxic mussels became toxic in a few days when transplanted to a toxic area but

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Wolff (1886) failed, understandably, to demonstrate toxicity in the water by injecting it directly into guinea pigs. Lindner (1888), although he did not recognize its significance, may have been observing the true source organism when he noted that the stomach contents of toxic mussels generally contained small, round, brown or brown-yellow 'infusoriencysten' that were not observed in mussels from non-toxic areas.

Similar objects were seen in California mussels by Stohler in 1930, although they were initially mis-identified as 'diatoms' (Sommer *et al.*, 1937). Despite the failure to detect toxicity in the particulates filtered from over 3,000 liters of sea water taken before, during, and after a period of high toxicity (Meyer, 1931), the Sommer and Meyer group persisted in thinking that food might be the source, in part because of the seasonal pattern of mussel toxicity and because toxicity occurred at the same time in another plankton feeder, the sand crab *Emerita analoga* (Sommer, 1932). Detailed plankton counts, begun in 1932, led to the discovery of *Gonyaulax catenella* (Whedon and Kofoid, 1936) which was abundant during periods of high toxicity. In July 1933, toxicity was finally demonstrated in the phytoplankton and, in June 1935, plytoplankton toxicity and *G. catenella* density rose to such levels that other sources of toxicity could reasonably be excluded. Laboratory feeding of mussels with phytoplankton *Gonyaulax* taken during this period indicated that mussels did indeed accumulate toxin from the phytoplankton (Sommer *et al.*, 1937). A similar organism, identified as *Gonyaulax tamarensis* (see section II.A.1 for discussion of the taxonomy)

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was found to be the source of toxicity in shellfish from the Bay of Fundy region (Needler, 1949; Prakash, 1963), extending south rather dramatically to the New England coast in 1972 (Bicknell and Walsh, 1975 and other references in LoCicero, 1975).

In an effort to explain increases in the toxicity of scallops (Bourne, 1965) and other shellfish (Needler, 1949; Prakash, 1963; Medcoff *et al.*, 1947) that did not appear to be due to motile *Gonyaulax*, it was suggested that a cyst form of *Gonyaulax* might be responsible, although the nature of *Gonyaulax* cysts was not properly understood. Subsequent studies have elucidated the nature and significance of cysts (Wall and Dale, 1968; Anderson, 1980) and have shown that cyst-rich sediments are toxic (Dale *et al.*, 1978), suggesting that they may be significant as a source of toxicity in shellfish (Yentsch and Mague, 1979).

Although there are some reservations (see below), the bulk of evidence points to these organisms, dinoflagellates from the section of the genus *Gonyaulax* assigned now to the new genus *Protogonyaulax* (Taylor, 1979; section II.A.1b), as the source of toxicity. This being the case, the ultimate causes of toxicity are the factors that control phytoplankton trophodynamics. Despite considerable effort, progress in the understanding of these mechanisms has been limited, some of the most promising results being the recognition (Nishihama, 1980; MacLean, 1977) that dinoflagellate populations may occupy discrete strata, often somewhat below the surface, such that surveys based largely on surface sampling may systematically overlook the bulk of the population.

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I.E. Mouse Assay

Despite repeated efforts to develop other methods, detecting and quantifying toxicity is still most efficiently and reliably done by intraperitoneal (ip) injection of toxin solutions into mice and relating the death time to an established dose response curve to determine the amount of toxin in the sample.

The injection of shellfish extracts to determine toxicity was reported by Salkowski (1885) and developed during the studies on California mussel toxicity (Prinzmetal *et al.*, 1932; Mueller, 1935; Sommer and Meyer, 1937) into a quantitative method using ip injection of mice. The standard unit initially used was a 'minimum lethal dose', evolving to a more convenient and only slightly higher 'dose lethal in 10-20 minutes'. This was eventually formalized to the current definition of a mouse unit as the amount killing a 20 gram mouse 15 minutes after ip injection of 1.0 mL solution, pH in the range 2 to 4. The dose response curve presented by Sommer and Meyer (1937) has been accepted as the basis for the standard method (AOAC, 1975; McFarren, 1959). Since it is found that death times in the range 5-7 minutes (corresponding to 1.92 to 1.39 mouse units) provide more precise results, samples with sufficient toxicity are diluted on the basis of trial injections to give death times in this range. The assay thus serves less as a quantitative detector used over its dynamic range, than as the end point of a titration, the magnitude of the result being determined primarily by volume ratios. Uncertainties about the slope of the dose-response curve are thus less of a problem than they might be.

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In samples of low potency, which call for little or no dilution, other substances can interfere seriously with the test. The presence of 1% sodium chloride in the injected solution reduces apparent toxicity by 50% (Schantz *et al.*, 1958). The apparent potency of solutions with a pH above 4 is substantially reduced (Wiberg and Stephenson, 1960; Schantz *et al.*, 1958).

To adjust for differences in technique and mouse sensitivity, results are normalized using purified standard shellfish poison (Schantz *et al.*, 1958) supplied by the U.S. Food and Drug Administration (FDA). In the past, toxicity results have been expressed as the equivalent weight of standard toxin, most commonly as micrograms per 100 grams of shellfish meat. The variety of toxins now evident, with significant differences in their specific toxicities, renders this practice somewhat misleading. The assay results obtained during this study are therefore expressed as 'standardized' mouse units, for which the raw data have been multiplied by a factor of 1.267. This value was established by summarizing 50 standardizations conducted over the period of the study with FDA standard toxin. From the same data the 95% confidence limits were estimated to be ± 23% of the mean, in good agreement with the generally stated precision of the test, ± 20% (AOAC, 1975). For ease of comparison, values cited from the literature that were obtained using the standard protocol have been converted from the equivalent weight of toxin to standard mouse units using the accepted value of 5500 mouse units per milligram of standard toxin.

While some of the variability in assay results can be attributed to that among mice, some clearly results from difficulties implicit in

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the technique of ip injection. Steward *et al.* (1968) gave 150 mice nominal ip injections of a radiological contrast medium and sacrificed them 15-30 minutes later. X-ray showed that in 21 all or part of the innoculum was somewhere other than in the peritoneal cavity.

I.F. Toxins

Salkowski (1885) and, later, the Sommer and Meyer group (Meyer *et al.*, 1928; Mueller, 1935) found that the material responsible for the acute effects of toxic shellfish was soluble in water and other highly polar solvents, insoluble in less polar solvents, relatively stable in acidic solution, and rapidly degraded in alkaline solution. Subsequent attention has been focused on this component of toxicity, which came to be known as paralytic shellfish poison. Early work on the chemistry of this material, based primarily on extracts of California mussels and Alaska butter clams, led to the isolation of a single substance called saxitoxin (Schantz *et al.*, 1966) and the general impression that toxicity along the Pacific coast was due only to this substance. Purification of saxitoxin was achieved using carboxylate cation exchange resins (Schantz *et al.*, 1957) to which the toxin bound and could be eluted well resolved from other material by dilute acid. Since it had been found that toxicity was lost at elevated pH, laboratory manipulations were generally conducted at a pH of 6 or below, conditions under which saxitoxin was eventually found to bear a net charge of +2, the apparent reason for its binding to carboxylate resins. Titration of saxitoxin under an inert atmosphere revealed two dissociable protons (Schantz *et al.*,

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1961) and demonstrated that the loss of toxicity from exposure to high pH was due to oxidation, rather than high pH *per se*.

This line of work culminated in 1975 with the determination of the correct structure of saxitoxin (1; see Figure 9, pg. 38) by x-ray crystallography of the di-parabromobenzenesulfonate salt (Schantz *et al.*, 1975) and, shortly thereafter, of the ethyl hemiketal (20; see Figure 11, pg. 40) (Bordner *et al.*, 1975).

As suitable methods were found for the purification of saxitoxin, it became apparent that toxicity in shellfish from the north Atlantic differed (Schantz, 1960; Evans, 1970), a large component not being retained on carboxylate resins as strongly as saxitoxin (1). Schantz (1960) hypothesized that the unretained material was less basic. Intensive study, prompted by the New England red tide of 1972, led to the development of methods for purifying this material, relying largely on the binding of the toxins to the polyacrylamide gel BioGel P2 for their initial recovery from extracts (Shimizu *et al.*, 1975b). Progress was greatly aided by thin layer chromatographic (TLC) techniques developed by Buckley (Buckley *et al.*, 1976), which remain the best means available for the resolution and detection of the toxins. From the resulting studies, several toxins were identified by chromatographic methods and named before their structural relationships were known. Structural studies have shown five of them to be the N-1-hydroxy and 11-hydroxy-sulfate derivatives of saxitoxin, discussed further in section II.B.

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I.G. Some Reservations

While the overwhelming weight of evidence points to saxitoxin and its derivatives as the active substances in shellfish toxicity and to gonyaulacoid dinoflagellates as the primary source, some reservations are in order. Because of the findings of Sommer and his associates, subsequent attention has been focused on the water-soluble component of toxicity which appears responsible for acute symptoms in human victims and experimental animals. This does not exclude the presence of other toxins of lower potency that might be masked by PSP. Such were indeed found by Sommer *et al.* (1937). Studies implicating *Gonyaulax* as the source (Sommer *et al.*, 1937; Needler, 1949) have generally acknowledged that they could not exclude the possibility of additional sources. Sommer not only recognized that non-living particulates might be a source, but demonstrated that substantial amounts of toxicity could be sorbed on beach sand (Sommer, 1932).

Finding saxitoxin ($\underline{1}$) and related substances in the freshwater blue-green alga (=cyanobacterium) *Aphanazomenon flos-aquae* (Jackim and Gentile, 1968; Alam *et al.*, 1978) shows that the production of the toxins is not restricted to gonyaulacoid dinoflagellates, if it occurs there at all. Notwithstanding the finding of toxicity in cultures of *Gonyaulax* claimed to be axenic (Burke *et al.*, 1960), Silva (196?) has suggested that toxicity in *Gonyaulax* is due to endosymbiont bacteria.

Although PSP in most organisms can be reconciled to some extent with toxicity originating in *Gonyaulax* and accumulating in filter feeders or in predators upon filter feeders, the relationship is not

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clear in some cases. Saxitoxin (1) and its derivatives have been found in Xanthid crabs from the south coast of Japan and islands to the south and west, the toxicity being restricted to a few species and reaching exceptionally high levels (Hashimoto *et al.*, 1969; Konosu *et al.*, 1969; Koyama *et al.*, 1981; Yasumoto *et al.*, 1981). The crabs appear to be neither predators nor filter feeders and the distribution of toxicity within and among individuals defies explanation at this point.

I.H. Paralytic Shellfish Poison in Alaska

Alaska has a vast resource of bivalve molluscs (Orth *et al.*, 1975), a wealth of habitat suitable for the cultivation of still more shellfish, and the distinction of what may have been the largest mass mortality from PSP in history (Fortuine, 1975). In 1799, a party of Aleut hunters left a newly-established settlement near Sitka and, a few miles to the northeast, along a channel now called Peril Straits, ate mussels. Over 100 people were reported to have died in a few hours. The accounts are sufficiently sketchy that one can be sure neither of the numbers nor that the cause was actually PSP, but the event seems quite plausible.

Bivalve fisheries eventually developed here, based mostly on razor clams in Southcentral and butter clams in Southeastern Alaska (SEAK). The butter clam industry grew rather briskly during WWII due to an increase in demand but came to a standstill in 1946 when the FDA detected toxicity with mouse bioassays of processed clams (Magnussen and Carlson, 1951). The industry found this hard to accept, particularly because there was little or no history of human illness from eating butter clams.

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However, the result should hardly have been surprising because Pugsley (1939), using the mouse bioassay, had found butter clams from around Prince Rupert to be toxic. In addition to being a generally profitable resource, the butter clam fishery was beneficial to the SEAK economy because activity could be concentrated in the off-seasons of the other, necessarily seasonal fisheries (Magnussen and Carlson, 1951).

Concern over the problem prompted studies at the Ketchikan Fisheries Technological Laboratory from 1946 to 1951 and intermittently in the years following. These established that toxicity was patchy, occurred over most of SEAK, and throughout most of the year (Chambers and Magnussen, 1950) so that it did not appear that the problem could be addressed by simple closures in certain areas or seasons.

Organisms resembling *Gonyaulax* have been found in Alaska both during surveys (Schantz and Magnussen, 1964; Neal, 1967; Chang, 1971) and in isolated episodes of PSP in False Pass (Meyers and Hilliard, 1955) and Tenakee (Zimmerman and McMahon, 1976). Neal (1967) was able to correlate toxicity in mussels near Ketchikan with the abundance of *Gonyaulax*. With this exception, however, the surveys were unable to find a correlation between toxicity in shellfish and the abundance of *Gonyaulax*, or generally reconcile the levels of toxicity found with the abundance of *Gonyaulax* on any occasion. There appeared to be far more toxin in the shellfish than could be obtained from the numbers of *Gonyaulax* found or, more generally, not found (Schantz and Magnussen, 1964). It should be noted, however, that the surveys were mostly limited to surface sampling and, on the basis of recent results (MacLean,

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1977; Nishihama, 1980) may have systematically missed a significant portion of the population.

It was particularly difficult to reconcile the close proximity of toxic and non-toxic shellfish beds (Schantz and Magnussen, 1964) with a water-born toxin source, given the amount of current action in SEAK. Neal (1967), referring to the suggestion by Bourne (1965) that Atlantic scallops might obtain toxin from *Gonyaulax* cysts, felt that sedimentary material might well help explain patterns of toxicity in butter clams and recognized that stirring mechanisms would be important if such were the case, but found no correlation between records of butter clam toxicity and wind velocity. Schantz and Magnussen (1964) also recognized the possibility that toxins could be supplied by sedimentary material.

Although the classical studies of toxin chemistry were based largely on toxin from butter clam siphons (e.g. Schantz *et al.*, 1957) and led to the conclusion that toxicity in Alaska was due primarily to saxitoxin (1), subsequent work found neosaxitoxin (7) as a minor component in butter clam siphons from Porpoise Island (Oshima *et al.*, 1977). Toxicity in mussels from Haines and Elfin Cove was found to be due primarily to derivatives of saxitoxin (Shimizu, 1978a). Reichardt subsequently demonstrated (Hall *et al.*, 1979) that a substantial component of toxicity in butter clams was due to substances distinct from 1 or 7.

In summary, the problems are that only a limited correlation has been found between the abundance of the presumed source organism and the levels, timing, and spatial distribution of toxicity in Alaskan shellfish, and that the toxin composition appears to differ from that previously recognized.

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I.I. Goals

The long term goals of this work are to define the metabolic origin and significance of the toxins, the nature of their selective binding, and their movement through food webs. The latter is with particular reference to developing marine food resources in Alaska, the first task being to remove toxicity as an impediment to the development of the Alaskan shellfish industry.

The immediate goals of this study were to culture the presumed toxin source organisms from Alaskan waters and to determine the kind and amount of toxin they contain.

Part II, which follows this section, describes the investigations. Several caveats appropriate in consideration of the results are summarized in section III. Section IV, which considers the toxins in relationship to the source organisms, to substrates, and to the food chain, is designed to provide a framework of general utility. It includes but goes somewhat beyond discussion of the present results.

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II. INVESTIGATIONS

II.A. The Organisms

II.A.1. Introduction

II.A.1a. General Description of *Protogonyaulax*

The organisms are most familiar as the motile, vegetative stage which is spheroidal, some strains tending to be notably oblate or elongate, with overall dimensions on the order of 20 to 50 microns. The protoplast is enclosed in a cellulose shell called the theca (Figures 1, 2) which is divided into two portions: the epitheca (anterior) and hypotheca (posterior) by an equatorial groove called the girdle. The hypotheca has a longitudinal groove, the sulcus, running from the girdle to the posterior end. Two flagella originate at the intersection of the girdle and sulcus, the transverse flagellum lying within the girdle and the longitudinal flagellum trailing aft along the sulcus. The theca is composed of about two dozen major polygonal plates, with several minor plates in the sulcus near the flagellar insertion and at the apex. The plates lack obvious decoration, one factor which permits facile discrimination between these and other dinoflagellates about the same size.

The shape and arrangement of plates, the traditional basis for taxonomic assignments, is obscured in whole cells by the cell contents and a proteinaceous membrane that covers the theca. Observations require removal of the covering and cell contents without disintegration of the theca and/or visualization of the sutures between the plates with staining or phase contrast microscopy.

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Figure 1. Empty theca of *Protogonyaulax* clone KNO3. Note plate detail in hypotheca (lower portion). Bar is 30 microns. Photo by Rita Horner.

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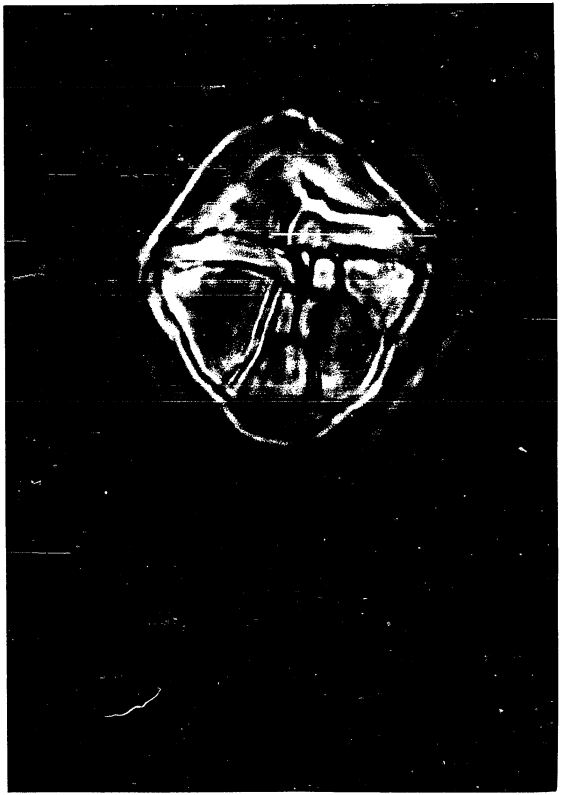

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Figure 2. Empty theca of *Protogonyaulax* clone HC01. Note plate detail in epitheca (upper portion). Bar is 30 microns. Photo by Rita Horner.

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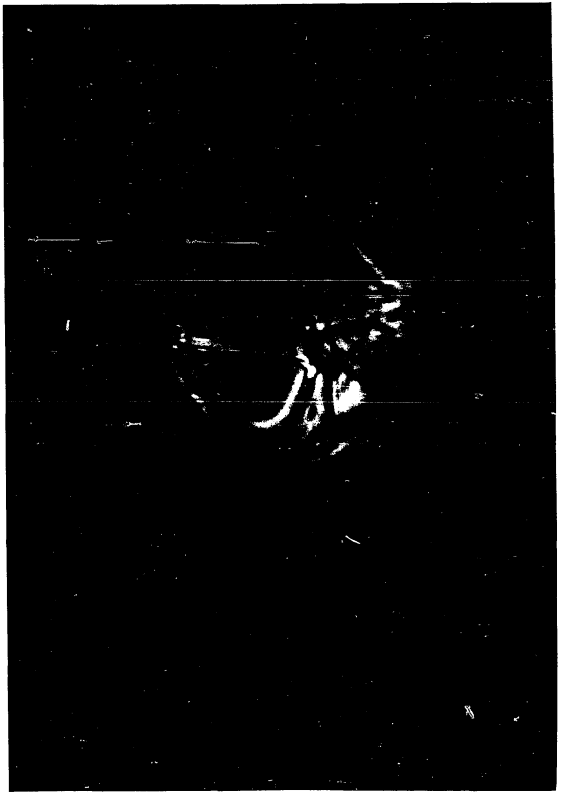

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Excellent scanning electron micrographs of thecal ultrastructure have appeared (Postek and Cox, 1976; Loeblich and Loeblich, 1975) but require sample preparation under conditions that prevent collapse of the theca, which is relatively delicate.

Cells ecdyse when exposed to stress, forming immotile 'pellicle cysts' (see below), regenerating their thecae and becoming motile again when acceptable conditions are restored.

Many *Protogonyaulax* tend to form chains, ranging from doublets (Figures 3, 4) to snakes of over 30 cells, which result from incomplete separation following division (Thomas, 1974). Division occurs on a diagonal plane, so that chains with recently divided cells have a characteristic 'Z' appearance.

The organisms are photosynthetic, with varying amounts of accessory pigments so that their color ranges through green, brown, and copper. They appear to be holophytic, no growth being observed in the dark with a variety of organic substrates (Norris and Chew, 1975; Yentsch *et al.*, 1975).

The nucleus is large, typically 'C'-shaped (Figures 5, 6).

II.A.1b. Taxonomy

The taxonomy of this group of dinoflagellates is best described as being in a state of flux. At the genus level it has long been recognized (Graham, 1942; Howell, 1953; Steidinger, 1971) that *Gonyaulax* is a heterogeneous group in dire need of revision, particularly that the toxic gonyaulacoids form a distinct subgroup having little in common with the

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Figure 3. *Protogonyaulax* clone EC06, chain of two cells. Fixed with Lugol's iodine. Bar is 30 microns. Photo by Rita Horner.

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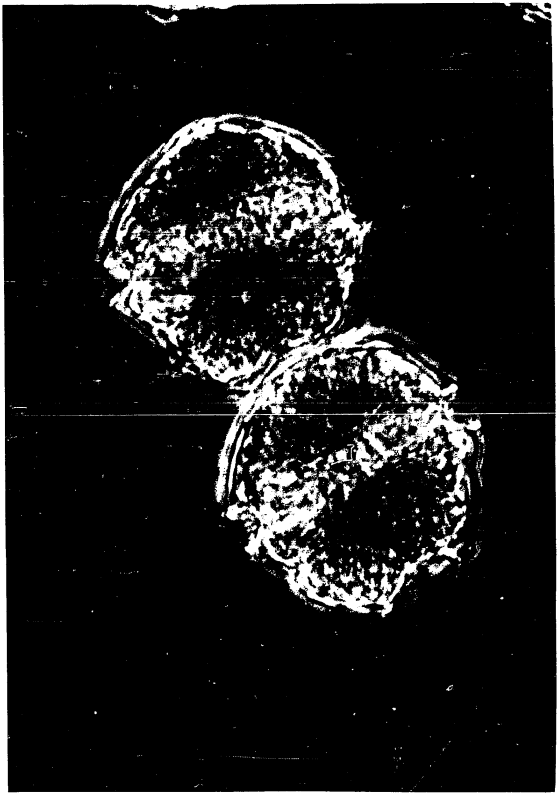

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Figure 4. *Protogonyaulax* clone EC06, chain of four cells. Fixed with Lugol's iodine. Bar is 30 microns. Photo by Rita Horner.

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Figure 5. Fuelgin stained *Protogonyaulax* nucleus. Note the characteristic 'C' shape. Histology and photo by George Mueller.

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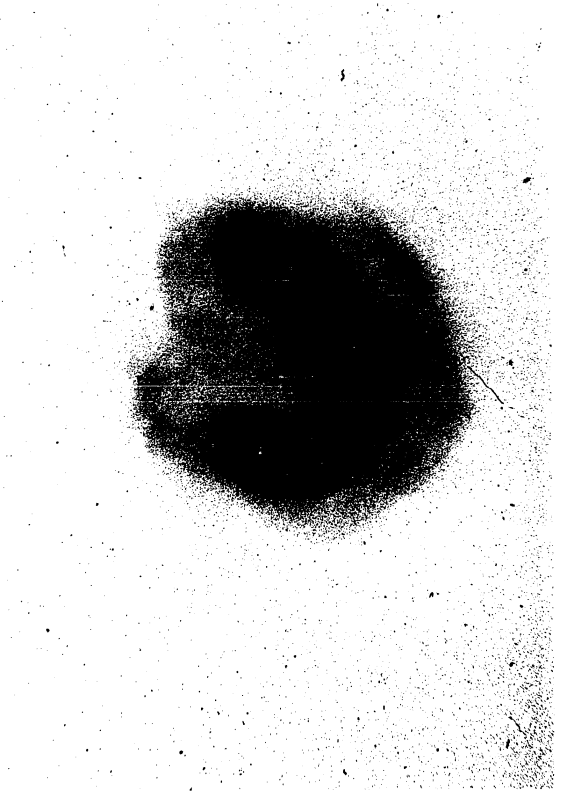

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Figure 6. Fuelgin stained *Protogonyaulax* nuclei shortly after division. Histology and photo by George Mueller.

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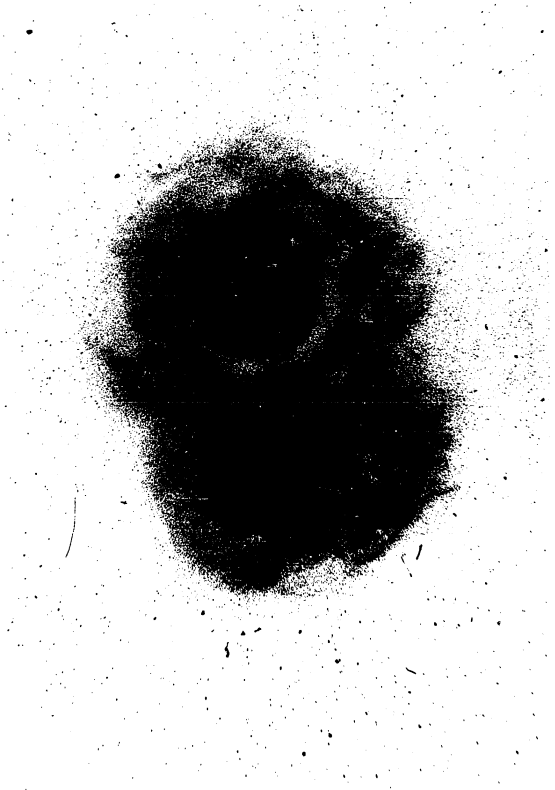

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other species. This problem has recently been addressed by both Loeblich and Loeblich (1979) and Taylor (1979). We follow the revision proposed by Taylor (1979), which transfers the species of interest to the new genus *Protogonyaulax*. In referring to other work in this thesis, however, the genus and species names used in the references cited are employed with the exception that the variant spelling '*Goniaulax*' is converted to '*Gonyaulax*'. For reasons that will become evident, the isolates obtained and studied in this work will not be referred to a species, being instead identified by the isolate designator (such as BC07, isolate number 07 from Bartlett Cove) or simply '*Protogonyaulax*'.

The following remarks summarize the history of the taxonomy of the group. A more detailed review may be found in Taylor (1975).

Lebour described *Gonyaulax tamarensis* with a brief diagnosis in her book *The Dinoflagellates of Northern Seas* (1925), stating that it was found only within the Tamar estuary. Her illustrations present the hypotheca properly, but the epitheca was apparently drawn optically reversed. Nevertheless, other workers found material in the Bay of Fundy (Gran and Braarud, 1935) and Norway (Braarud, 1945) that they assigned to *G. tamarensis*, providing figures now considered to be correct without remarking on the difference.

In the course of studies on shellfish toxicity in California, Whedon and Kofoid (1936) described two new species, *Gonyaulax catenella* and *Gonyaulax acatenalla*. The two are very similar in the arrangement of plates, the differences lying in the tendency of *G. catenella* to form

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long chains, the profile of the cell, and the shape of individual plates. *Gonyaulax catenella* was established to be the prime source of toxicity in California mussels (Sommer *et al.*, 1937). *Gonyaulax acatenella* was not correlated with toxicity at the time, nor was it reported again until an outbreak of PSP in British Columbia where toxicity in shellfish resulted from a dense bloom identified as *G. acatenalla* (Prakash and Taylor, 1966). Extraction of phytoplankton samples revealed a toxicity of 170 micromouseunits per cell.

In their description of *G. acatenella*, Whedon and Kofoid (1936) note its similarity to *G. tamarensis* and relate comments by Lebour to whom they submitted material for comparison. She considered the two to be similar in most respects, differing only in the shape of some of the apical plates. Given the similarity of plate arrangement in *G. catenella* and *G. acatenella*, these comments would seem to verify that the plate configuration of *G. tamarensis* from the type location was as generally accepted and that the original drawing presented by Lebour was indeed partly reversed. Prakash and Taylor observed short chains in their *G. acatenella* material and remarked on the possibility that *G. catenella* and *G. acatenella* might be conspecific.

Following the work of the Sommer and Meyer group, Needler (1949) established that toxicity in shellfish in the Bay of Fundy area correlated with the abundance of *G. tamarensis*. Her comments appear to imply that she submitted material to Lebour for identification. Some years later Prakash (1967) extended Needler's work, culturing *G. tamarensis* from the Bay of Fundy and demonstrating it to be toxic. However, he

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also cultured strains of *G. tamarensis* from the type location and found them to be non-toxic.

Studying the taxonomy of the group following the New England red tide of 1972, Loeblich and Loeblich (1975) attempted to resolve the disparity between the non-toxicity of *G. tamarensis* from the type location and the toxicity of other material by establishing the species *Gonyaulax excavata*, based on a description by Braarud (1945) of an isolate from Norway. This proposal was withdrawn, however (Schmidt and Loeblich, 1979a), when it was recognized that the criteria used for the separation of the species occurred in a number of combinations that did not support species distinction.

Other species have been proposed by Hsu (1967) and Balech (Taylor, 1975) that may eventually prove relevant to the populations found in the northeast Pacific, but which have not yet found broad acceptance.

II.A.1c. Cysts and the Life Cycle

Protogonyaulax are most commonly recognized as the vegetative stage, the motile, thecate form that predominates in actively growing cultures. When such cells are stressed, however, they rapidly ecdyse to form immotile, globular cells referred to by Anderson and Wall (1978) as pellicle cysts. The formation of pellicle cysts has been found to result from temperature stress (Dale, 1977; Prakash, 1967; Anderson and Wall, 1978), copper toxicity (Anderson and Wall, 1978; Anderson and Morel, 1978), and nutrient starvation (Anderson and Wall, 1978). Forms that were likely pellicle cysts have commonly been observed in cultures

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that were probably old or unhealthy (Hsu, 1967; Prakash, 1967; Dupuy, 1968, among others). Although their viability decreases with time, pellicle cysts may remain viable for weeks, forming a new theca and becoming motile again when the stress is relieved (Anderson and Wall, 1978). To past workers it seemed reasonable that *Protogonyaulax* should have a resting stage and, given that pellicle cysts were the all too familiar result of sub-optimal culture conditions, there are many early allusions to them as the apparent resting stage.

The true resting stage was recognized only in the mid-1960's, as discussed by Wall and Dale (1968). The present concept of the *Protogonyaulax* life cycle has evolved largely from the work of Anderson and Wall (1978) and Anderson (1980). Some evidence for sexuality has been provided by Turpin *et al.* (1978).

The cycle includes six stages:

1) vegetative: motile, thecate cells with two flagella arising ventrally at the juncture of the girdle and sulcus, one transverse and the other trailing.
2) gamete: resembling vegetative cells but slightly smaller.
3) planozygote: resulting from the fusion of gametes, posteriorly biflagellate, slightly larger than vegetative cells.
4) hypnozygote: the 'resting cyst', which has an obligatory period of dormancy that varies with conditions. Hypnozygotes are stable for well over a year and germinate primarily as a result of temperature change, most commonly an increase from a low *in situ* temperature. They are elongate, agglutinous, and develop a conspicuous pigmented spot (Figure 7).

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Figure 7. A *Protogonyaulax* hypnozygote or resting cyst. The egg-shaped, agglutinous cyst is typically found encased in sediment particles which have been partially removed here by gentle sonication. Photo by George Mueller.

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5) planomeiocyte: the motile cell that emerges from the hypnozygote. It retains the pigmented spot and elongate profile of the hypnozygote and is thus distinct from subsequent vegetative cells.
6) pellicle cysts, the formation of which appears to be a strategy for responding to temporary stress.

II.A.2. Isolations

The failure, on a preliminary expedition to Southeast Alaska, to find *Protogonyaulax* in the phytoplankton, followed by a chance encounter with a classic paper on the incubation of dinoflagellate cysts (Wall *et al.*, 1967) led to the idea of using benthic cysts as the source of cultures for this study. Their method was simplified by diluting sediments with medium and incubating the mixture, which circumvented the need to find and isolate the cysts themselves. The resulting motile cells, which generally appeared after 2 to 7 days incubation at room temperature and were far easier to see than cysts, were isolated by micropipet. Sediment samples were obtained from several locations along the Alaskan coast and, in all but a few cases, gave rise to motile *Protogonyaulax*. The success of this strategy was due in part to the stability of the cysts when refrigerated and in part to the generosity of other workers in providing samples. The locations from which the samples were obtained that gave rise to *Protogonyaulax* are summarized in Figure 8 and detailed in Table 1.

On a subsequent expedition to Haines in the summer of 1979, *Protogonyaulax* was finally encountered in one of several plankton tows. This material was the source of clones HG26, HG27, and HG29.

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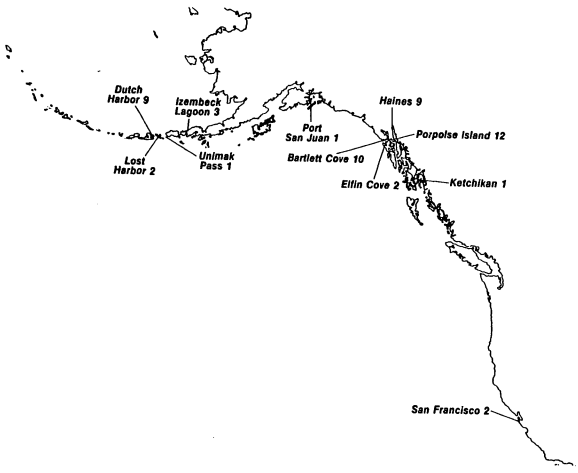

Figure 8. Locations sampled and the number of toxic strains isolated from each location.

Table 1. Toxic isolates.

Location	Coordinates Latitude N	Longitude W	Depth (m)	Comments	Isolate name
Southwestern					
Dutch Harbor	53°56.0'	166°34.0'	80		DH01
	53°53.35'	166°34.0'	51		DH02
					DH03
	53°53.1'	166°34.1'	48		DH04
					DH05
	53°53.27'	166°33.4'	58	a	DH06
				a, e	DH07
					DH08
					DH09
Lost Harbor	54°13.5'	165°36.5'	44	a, e	LH01
					LH02
Unimak Pass	55°07.1'	163°29.6'	28		UP01
Izembek Lagoon	55°53.78'	162°16.02'	1		IZ01
	55°53.78'	162°16.02'	1		IZ03
	55°53.82'	162°16.00'	1		IZ02
Prince William Sound					
Port Benny	60°03.0'	148°00.3'	30		PW05
				e	PW06
Icy Straits					
Elfin Cove	58°11.59'	136°20.54'	6		EC04
	58°11.34'	136°20.27'	4	e	EC06
Bartlett Cove	58°27.5'	135°53.5'	20	a	BC01
	58°27.4'	135°53.2'	9	a	BC02
					BC04
				a	BC10
					BC11
					BC12
	58°27.7'	135°53.2'	21	e	BC07
					BC08
					BC09
	58°27.5'	135°53.0'	10		f1501
					f1504
					f1506
					f1515

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Table 1. Continued.

Location	Coordinates Latitude N	Longitude W	Depth (m)	Comments	Isolate name
Porpoise Island	58°19.72'	135°27.3'	40		PI01
					PI13
					PI14
	58°19.32'	135°26.6'	43	e	PI07
					PI03
					PI12
					PI08
					PI09
					PI10
					PI11
					PI15
					PI26
Haines					
Flat Bay	59°09.0'	135°20.7'	d	e	HG01
					HG02
					HG10
					HG12
					HG19
	59°09.0'	135°20.7'	d		HG22
Taiyasanka Harbor	59°17.8'	135°25.9'		b	HG26
				b, e	HG27
				b	HG29
Ketchikan					
Boca de Quadra	55°19.4'	130°29.2'	91	e	KN03
California					
San Francisco	37°10.7'	122°23.6'	c		SF01

Notes:

a) Except for these six isolates, all strains are clonal, having been isolated as a single cell either initially or at some point early in the strain's history. For these six, the smallest unit isolated was a chain of two cells. None of my observations suggest that such a chain can arise by any means other than the division of a single cell. Such isolates are, in all probability, therefore clonal.

b) Thecate cells isolated from a plankton tow.

c) Sample filtered from a running seawater system that draws from the intertidal. Inspection shortly after collection revealed no motile dinoflagellates in this sample, but motile cells appeared following incubation. Given these circumstances, the motile cells probably arose from cysts.

d) Sample collected from the intertidal.

e) Thecae examined in sufficient detail to confirm assignment to *Protogonyaulax*. Cultures analyzed for toxin composition (Section II.C.4).

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Because of procedures used during isolation, some strains have unequivocally distinct parentage while others might possibly have arisen from the same cyst. In Table 1, isolates that may possibly be sympatric are grouped, while those that have unequivocally distinct parentage are separated by spaces.

Although it is not certain that the isolates obtained by sediment incubation came from resting cysts, it seems most likely that they did in view of the history of the samples and other observations. Objects corresponding to the descriptions of *Protogonyaulax* hypnozygotes were found in sieved and sonicated sediments from Porpoise Island that incubation experiments suggested were rich in cysts. One of these is shown in Figure 7. The motile cells that initially appeared in the incubation plates were often elongate and contained little pigmentation except for a single reddish spot, consistent with the description of *Protogonyaulax* planomeiocytes (Anderson, 1980). That the motile cells arose from cysts was further indicated by a set of incubation plates in which one was exposed to the normal conditions of light and room temperature, another was held at the same temperature in the dark, and a third was returned after dilution to 4°C in the dark. Motile cells were not apparent in the normal plate for the first two days, but then appeared and continued to appear for about a week. Motile cells appeared in the dark, warm plate at about the same abundance and for the same period while none appeared in the refrigerated plate. These responses are consistent with the behavior of *Protogonyaulax* resting cysts (Anderson, 1980).

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II.A.3. Toxicity of Cultured Cells

Pipet-isolated cells were grown to sufficient volume for the determination of toxicity by mouse bioassay. All the isolates included in Table 1 were assayed and found to be toxic. All were assigned to *Protogonyaulax*. For ten of these strains, also evaluated for toxin composition (section II.C.4), thecae were examined with the assistance of Dr. Rita Horner in sufficient detail to confirm assignment to *Protogonyaulax*. For the remainder of the isolates, assignment to *Protogonyaulax* was based on gross morphology, lateral profile, and chain formation. These assignments are probably correct, though hardly rigorous. In addition to these, 8 other clones, distinguishable from *Protogonyaulax* under the dissecting microscope, were assayed and found not to be toxic. One isolate, which grew poorly and died out before further work could be attempted and which did not seem to be *Protogonyaulax* but was similar enough that it could not be rigorously excluded, proved not to be lethal in the mouse bioassay. Beyond this one ambiguous case, no evidence was found for organisms resembling *Protogonyaulax* that were not toxic.

It is interesting to note that, in the incubations, there were few large, round dinoflagellates other than those that were assigned to *Protogonyaulax* and which, when tested, proved to be toxic.

Efforts to assign the isolates to species were exasperating. As will be suggested by the data in section II.C.4, it appears that established species do not suffice to describe the populations of *Protogonyaulax* found in the northeast Pacific.

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The plate tabulations of the familiar species *P. tamarensis* and *P. catenella* do not differ substantially, if at all, discrimation of the two being primarily on the basis of cell profile and the tendency to form chains. *Protogonyaulax catenella* was described by Whedon and Kofoid (1936) to be oblate and form long chains. Populations from Puget Sound (Norris and Chew, 1975) form chains of well over 16 cells. *Protogonyaulax tamarensis* is round and elongate, forming doublets but seldom longer chains (Taylor, 1979).

Routine observations for over two years of the isolates obtained during this study have provided a substantial body of qualitative impressions on cell profile, chain formation, and the variability of these properties within a clone. While there are isolates that, on the basis of profile or chain formation, might be assigned to one or the other of these species, the majority of the strains are intermediate or the properties sufficiently variable that assignment does not seem meaningful. The problem remains open.

II.A.4. Toxicity and Cyst Abundance in Porpoise Island Sediment

From qualitative observations of the sediment incubation plates it became apparent that there were substantial variations in the abundance of viable cysts from one location to another. This prompted a preliminary quantitative study of sediment toxicity and cyst abundance near Porpoise Island, which has a tradition of high PSP levels (Anon., 1962; Chang, 1971) and, from incubation experiments, seemed to have sediments rich in cysts.

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On the basis of quantitative dilution plates incubations, surface sediment collected from 40 m depth off the east shore of Porpoise Island (58°19.72'N, 135°27.3'W) on 25 March 1979, sieved and incubated starting 23 June 1978, appeared to have about 200 viable cysts per milliliter sediment. From the nature of the determination the limits of uncertainty are rather broad, but it is unlikely that the true concentration was less than 20 or more than 400 cysts/mL. Mouse bioassay of an extract from a portion of the sieved sediment suggested a concentration of 0.2 mouse units per milliliter of the original sample. A similar sediment incubation study from the same area, based on four grab samples taken the following year, supported the estimate of about 200 cysts/mL. Combining the estimates for toxicity and cyst abundance and assuming that the toxin is contained in the cysts yields a toxin content of about 1000 micromouseunits/cyst, on the same order as the upper limit of toxin content in motile cultured cells of clone PI07 (section II.C.2) which originated in the same area. It is also on the same order as the toxin content (680 micromouseunits/cyst) reported by Dale *et al.* (1978) for a sediment sample from the coast of Maine, rich in *Gonyaulax excavata* cysts.

II.B. Toxins

II.B.1. General Description of the Saxitoxins

For want of a better term, the family of substances structurally related to saxitoxin is referred to here as the saxitoxins. The array of twelve saxitoxins, herein suggested to represent the complete set

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extractable from *Protogonyaulax* is shown in Figure 9 and their functional relationships summarized in Figure 10. Other derivatives, some shown in Figure 11, can be produced in the laboratory (Ghazarossian, 1977; Boyer, 1980; Koehn *et al.*, 1981) and still others likely exist in nature (see section II.B.7). Furthermore, some properties of the toxins observable *in vitro* strongly suggest that the nature or environment of the toxins *in vivo* somehow differ. However, the array presented has a certain aesthetic appeal and accounts for the available evidence.

The natural occurrence of saxitoxins differing from the parent compound 1 had been suggested by the work of Schantz (1960) and Evans (1970) and was proven by the work of Buckley (Buckley *et al.*, 1975) and Shimizu *et al.* (1975a,b). Ghazarossian *et al.* (1974) suggested that the new compounds might be N-oxides; this suggestion was verified in a sense when Shimizu *et al.* (1978) found neosaxitoxin to be N-1-hydroxysaxitoxin, 7. Boyer *et al.* (1978) and Boyer (1980) established the structures of the epimeric 11-hydroxysaxitoxin sulfates 3 and 5. Subsequent work (Boyer, 1980; Fix Wichmann *et al.*, 1981a; Shimizu and Hsu, 1981) demonstrated that the two modifications can occur together, forming 9 and 11.

The nature and significance of the third modification, substitution of a sulfo group on the carbamoyl nitrogen forming 2, 4, 6, 8, 10, and 12, was first elucidated in the present work, although substances apparently corresponding to 4 and 6 have been obtained from cultured *Gonyaulax excavata* from Ofunato Bay, Japan (Oshima and Yasumoto, 1979), cultured *Gonyaulax tamarensis* (Ipswich strain) from New England

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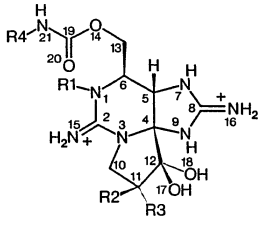

	R1	R2	R3	R4	
1	H	H	H	H	STX
2	H	H	H	SO_3^-	B1
3	H	H	OSO_3^-	H	GTX 2
4	H	H	OSO_3^-	SO_3^-	C1
5	H	OSO_3^-	H	H	GTX 3
6	H	OSO_3^-	H	SO_3^-	C2
7	OH	H	H	H	NEO
8	OH	H	H	SO_3^-	B2
9	OH	H	OSO_3^-	H	GTX 1
10	OH	H	OSO_3^-	SO_3^-	C3
11	OH	OSO_3^-	H	H	GTX 4
12	OH	OSO_3^-	H	SO_3^-	C4

Figure 9. The twelve *Protogonyaulax* neurotoxins.

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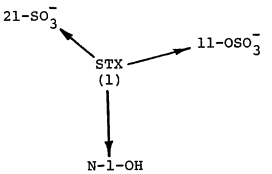

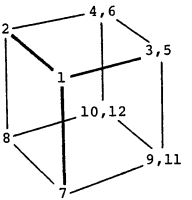

Figure 10. Functional group relationships among the saxitoxins.

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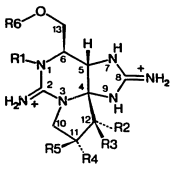

	R1	R2	R3	R4	R5	R6
13	H	OH	H	H	H	$CONH_2$
14	H	H	OH	H	H	$CONH_2$
15	H	OH	OH	H	H	H
16	OH	OH	OH	H	H	H
17	H	OH	H	H	H	H
18	H	OH	OH	H	H	$OCOCH_2CH_2COOH$
19	H	H	H	H	H	$CONH_2$
20	H	OH	OCH_2CH_3	H	H	$CONH_2$
21	H	OH	OH	OH	H	$CONH_2$
22	H	OH	OH	H	OH	$CONH_2$
23	H	OH	OH	OSO_3^-	H	H
24	H	OH	OH	H	OSO_3^-	H

25

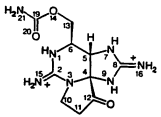

Figure 11. Other saxitoxin derivatives.

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Table 2. Abbreviations and names for saxitoxin derivatives.

Compound	Abbreviation	Common name	Semi-systematic name
1	STX	saxitoxin	saxitoxin
2	B1		21-sulfosaxitoxin
3	GTX 2	gonyautoxin 2	11α-hydroxysaxitoxin sulfate
4	C1		21-sulfo-11α-hydrosaxitoxin sulfate
5	GTX 3	gonyautoxin 3	11β-hydroxysaxitoxin sulfate
6	C2		21-sulfo-11β-hydroxysaxitoxin sulfate
7	NEO	neosaxitoxin	N-1-hydroxysaxitoxin
8	B2		21-sulfoneosaxitoxin
9	GTX 1	gonyautoxin 1	11α-hydroxyneosaxitoxin sulfate
10	C3		21-sulfo-11α-hydroxyneosaxitoxin sulfate
11	GTX 4	gonyautoxin 4	11β-hydroxyneosaxitoxin sulfate
12	C4		21-sulfo-11β-hydroxyneosaxitoxin sulfate
13	αSTOH		α-saxitoxinol
14	βSTOH		β-saxitoxinol
15	dcSTX		decarbamoylsaxitoxin
16	dcNEO		decarbamoylneosaxitoxin
17			decarbamoyl α-saxitoxinol
18			decarbamoylsaxitoxin hemisuccinate
19			12-deoxosaxitoxin
20			saxitoxin 12β-ethyl emiketal

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Table 2. Continued.

Compound	Abbreviation	Common name	Semi-systematic name
21			11α-hydroxysaxitoxin
22			11β-hydroxysaxitoxin
23			decarbamoyl 11α-hydroxysaxitoxin sulfate
24			decarbamoyl 11β-hydroxysaxitoxin sulfate
25			ketone form of saxitoxin

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(Kobayashi and Shimizu, 1981), and both oysters and cultured *Protogonyaulax catenella* from Senzaki, Japan (Onoue *et al.*, 1981b). The report of the 21-sulfo toxins from oysters is rather important, being the first direct demonstration that they occur under natural circumstances and are not merely induced by culture conditions.

Titrations of saxitoxin (1) by Schantz *et al.* (1961) revealed two dissociable groups, with pKa's 8.1 and 11.5. A more recent study (Rogers and Rapoport, 1980) has reported the values 8.22 and 11.28. Similar titrations of neosaxitoxin (7) revealed a third dissociable group, the pKa's being 6.75, 8.65, and 11.65 (Shizimu, 1978b). Studies of the variations in chemical shifts with pH in ^{13}C-NMR (Rogers and Rapoport, 1980) and ^{1}H-NMR (Shimizu, 1978b; Shimizu *et al.*, 1981) indicate that the pKa at about 11.5 corresponds to dissociation of the C-2 guanidinium, that at about 8.5 to the C-8 guanidinium, and the pKa 6.75 found for 7 to dissociation of the N-1-hydroxyl.

Because of the reputed susceptibility of the toxins to oxidation at elevated pH, manipulations have generally been conducted in acidic solutions wherein the dissociable groups are protonated, 1 and 7 being dications and binding strongly to carboxylate cation exchange resins. The 11-hydroxysulfate substituent found on 3, 5, 9 and 11 reduces their net charge to +1 (Boyer *et al.*, 1978; Boyer, 1980; Fix Wichmann *et al.*, 1981) under these conditions and accounts for their relatively weak binding to carboxylate resins. In the present study, the negative charge of the third substituent, found to be the 21-sulfo group, was originally inferred from chromatographic evidence. The charge

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relationships of the twelve toxins under acidic conditions are summarized in Figure 12a.

Titrations for toxins other than 1 and 7 have not been reported and the indirect data, from which their charge and dissociation constant might be inferred, are mixed. The question is of particular interest because in the pH range likely to be of physiological significance (around 7 to 8), the N-1-hydroxyl might, or might possibly not, be largely dissociated. If it is, leading to the charge relationships summarized in Figure 12b, then 8, 9, and 11 will, like 4 and 6, bear no net charge, posing an important constraint on models of toxin binding in shellfish and excitable membranes (section IV.B.2). Chromatographic evidence (sections II.B.3 and 4) suggests that 8, 9, and 11 are uncharged at pH 7.5 and that the charge of 10 and 12 is altered at pH 8. On the other hand, electrophoretic data reported by Shimizu and his associates (Fallon and Shimizu, 1977; summary in Shimizu, 1979) indicate that 9 still has a partial net positive charge at pH 8.7. The question invites further study.

Toxins bearing the 11-hydroxysulfate substituent epimerize readily *in vitro*, the 11α epimer predominating in equilibrated mixtures in each case (section II.B.6a; Boyer *et al.*, 1978; Boyer, 1980; Fix Wichmann *et al.*, 1978a,b). It is important to note that this is in marked contrast to the distribution found in dinoflagellate extracts, where the 11β epimers predominate. This relationship suggests 1) that the nature or circumstances of the toxins *in vitro* somehow differ and, 2) that the alpha epimers may not be present in the source organism, arising only through epimerization once the cell is disrupted.

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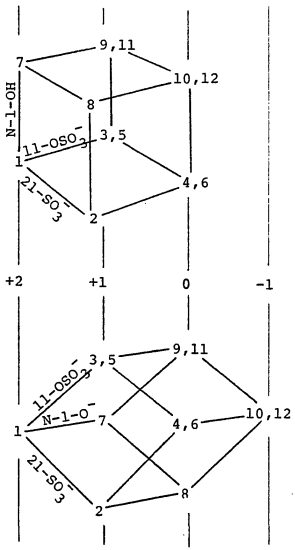

Figure 12a (upper). Charge relationships among the saxitoxins below about pH 6.

Figure 12b (lower). Charge relationships among the saxitoxins following deprotonation of the N-1-hydroxyl, likely prevailing in the pH range 7-8.

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Toxins bearing the 21-sulfo (or carbamoyl-N-sulfo, or sulfamate) substituent are hydrolyzed under relatively mild conditions (0.1 M HCl, 100°C, 5 minutes; section II.B.6a and Hall *et al.*, 1980) to form the 21-H (or carbamate) toxins. Hydrolysis of the 11-hydroxysulfates 3 and 5 under stronger conditions (2.5 M HCl, 90°C, 90 minutes) yields the 11-hydroxy saxitoxins 21 and 22 (Boyer *et al.*, 1978; Boyer, 1980), while even stronger conditions (7.5 M HCl, 100°C, 7.5 hours) hydrolize the carbamoyl group from 1 to give 15 (Ghazarossian *et al.*, 1976) or from 7 to give 16 (Koehn *et al.*, 1982).

Shimizu has demonstrated that the N-1-hydroxyl and the 11-hydroxy-sulfate groups can be removed from 3, 5, 7, 9, and 11 both by treatment with zinc dust and dilute HCl (Shimizu and Hsu, 1981), and by incubation with scallop tissue (Shimizu and Yoshioka, 1981).

In the present study, discovery of the 21-sulfo toxins resulted from the pursuit of two observations with extracts from clone PI07:

1) As had first been noted by Proctor (1973), cell-free extracts of *Protogonyaulax* increase in toxicity when heated with hydrochloric acid. The increase in potency following this treatment is referred to here as 'Proctor enhancement', and is discussed in section II.B.2.

2) A substantial portion of the toxicity did not bind to chromatographic columns under conditions that bound the known toxins. Following the treatment that resulted in Proctor enhancement, all the toxicity could be bound.

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II.B.2. Proctor Enhancement

The potencies of extracts, expressed as the apparent toxin content per cell, obtained under a variety of extraction conditions are shown in Table 3. Potency reached a plateau following heating for 5 minutes with hydrogen ion concentrations over about 0.09 M. Longer heating or higher acidity caused little change. In another series of comparisons the potencies of solutions 0.1 M in HCl after 2.5, 5 and 10 minutes heating differed little, indicating that 5 minutes at 100°C was adequate. However, the potencies of samples heated 5 minutes that had been prepared with 0.02 and 0.04 M HCl were substantially below that prepared with 0.1 M HCl, indicating that 0.1 M was marginal. In subsequent work, the HCl concentration of samples being prepared for assay was therefore adjusted, with consideration for the buffer capacity of the sample, to assure an *in situ* hydrogen ion concentration in the range 0.1 to 0.4 M. Preliminary experiments had shown that the results were not altered by the presence or absence of cellular debris, so the effect is not merely an increase in the efficiency of extraction.

While the above conditions were required to assure prompt enhancement to a plateau value of toxicity, slow increases occurred in samples held at room temperature and at lower acidity. It was therefore apparent that reliable estimates of potency could usually be obtained only following treatment to give this plateau value. Unless otherwise noted, the toxicities of cells, extracts, and chromatographic fractions were determined following such treatment.

Proctor enhancement, the increase in potency with such treatment, is an observable property. It appears to be due to hydrolysis of the

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Table 3. The potency of extracts prepared with different periods of heating and concentrations of hydrochloric acid.

	Period of heating, minutes in 100°C water bath				
[HCl], M	0	1	5	10	25
0.01				134	
0.09				402	
0.10	91	251	412		390
1.1				419	

Values shown are the toxicity in micromouseunits per cell in the original sample.

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21-sulfo group, apparent from TLC (section II.B.6a) and the potencies of purified toxins (Figure 20) before and after hydrolysis.

II.B.3. Chromatography

II.B.3a. BioGel P2

Chemical studies of the toxins, based primarily on extracts from clone PI07, began to progress when it was found, contrary to expectations, that the toxicity of extracts applied to a column of BioGel P2 emerged after (and well resolved from) most of the non-toxic constituents when eluted with 0.1 M acetic acid. The purity and concentration of the fractions were such that they could be applied directly to TLC plates, developed, and visualized using the methods of Buckley (Buckley *et al.*, 1976). In practice, ionic constituents were located by monitoring conductivity while the toxins were located with the Buckley spot test (section II.B.3d). One such run for clone PI07 is shown in Figure 13. A standard chromatogram, run under the same conditions, employing a mixed standard with 2 micromoles of each of the toxins except $\underline{\underline{10}}$ and $\underline{\underline{12}}$, is shown in Figure 14. It should be noted that the elution volumes, which are rather consistent for extracts, differ in the standard of pure toxins. However $\underline{\underline{1}}$, $\underline{\underline{3}}$, and $\underline{\underline{5}}$, added to an extract of PI07, which does not contain them, elute at the positions in which they would normally appear in extracts that do contain them. Elution volume in this system thus appears to be controlled by matrix effects which are apparently sufficiently uniform in dinoflagellate extracts that consistent results are obtained.

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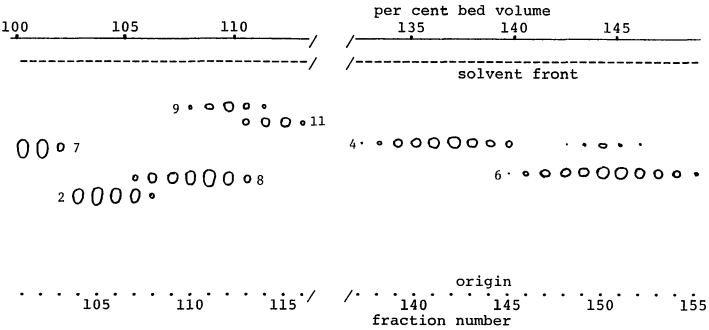

Figure 13. Chromatography of an acetic acid extract of *Protogonyaulax* clone PI07 on BioGel P2. Column was eluted with 0.1 M aqueous acid. Fractions from the column were spotted on TLC plates and developed for three hours to give the pattern shown. The figure was traced from a photograph of the TLC plates, taken under 366 nm UV light, following visualization as described in the Experimental section. The toxins are identified on the profile by number.

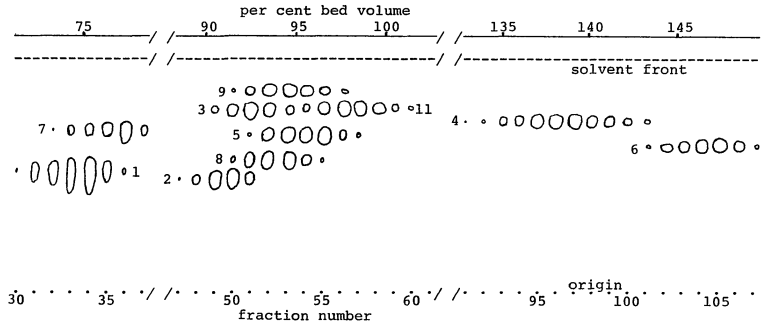

Figure 14. Chromatography of a mixed standard of pure toxins on BioGel P2. Mixture contained 2.0 micromoles of each toxin except 10 and 12. Conditions correspond to those in Figure 10. The toxins are identified on the profile by number.

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The toxins elute as three clusters:

Group A, including 1 and 7

B 2, 8, 3, 5, 9, and 11; and

C 4, 6, 10, and 12.

On the basis of electrophoresis and other chromatographic evidence, these correspond to toxins bearing a net charge of +2, +1, and 0 respectively. It is interesting to note that the elution positions of 4 and 6, which differ little in extracts or in the mixed standard, are also essentially the same on BioGel P2 columns eluted with water while the group A and B toxins are bound, eluting with acetic acid.

Resolution of the group C toxins 10 and 12 from 4 and 6 was achieved by elution from BioGel P2 with pH 8 ammonium acetate buffer, 10 and 12 emerging earlier than 4 and 6.

BioGel P2 has been extensively used in toxin purifications (Shimizu, 1975b; Buckley *et al.*, 1975), most commonly as an initial purification step in which the toxins are expected to remain bound on the column when applied at about pH 5 and washed with water. It is important to recognize that the group C toxins are not bound under these conditions, despite the fact that they are the last to elute under isocratic conditions with 0.1 M acetic acid.

II.B.3b. Carboxylate Cation Exchange Resins

Carboxylate cation exchange resins have played an important part in the chemistry of the toxins, being used for the first purifications of saxitoxin (1) (Schantz *et al.*, 1957) and more recently for analytical

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separations of saxitoxin derivatives (Shimizu *et al.*, 1975b; Shimizu, 1978a). Several products have been used. Those manufactured by Rohm and Haas, designated IRC50, CG50, XE64, and IRP64 are apparently much the same material, a methacrylic acid-divinylbenzene polymer, in different states of refinement and particle size. BioRad Laboratories, the supplier of another called BioRex 70, states that their product has similar properties. In the present study, BioRex 70 and IRP64 were both used, with no differences in properties being apparent. Subsequent references will be simply to 'carboxylate resins'.

Carboxylate resins were used mostly for further purification of toxic fractions obtained from preparative runs on BioGel P2 which, depending on the degree of overload, were the more or less separated groups A, B, and C. When applied under proper circumstances to carboxylate resins in the hydrogen form (H-carboxylate), group A and B toxins bound and were eluted well resolved from each other with dilute acetic acid. Group C toxins were neither bound nor apparently retained, passing through the column with other unretained material in the water wash.

The group B toxins, and most non-toxic bound material, were eluted by acetic acid at concentrations below 0.1 M. Resolution within the group and from other material was improved only slightly by using shallow acetic acid gradients, such as that shown in Figure 15.

The group A toxins were eluted promptly by 1 M acetic acid or following prolonged elution (several bed volumes) with 0.1 M acetic acid. Neosaxitoxin (7) emerged first, partially resolved from saxitoxin (1).

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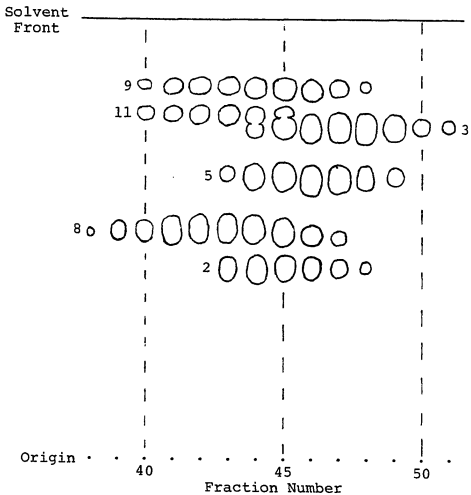

Figure 15. Elution of group B toxins from the carboxylate resin IRP64. Bed prepared in the hydrogen form. Ammonium hydroxide (10 mmol) was applied and the column washed with water prior to application of the sample. The column was then eluted with 1 bed volume of water followed by an acetic acid gradient from 0.05 to 0.1 M over 3 bed volumes. The toxins emerged with the last third of the gradient. The figure is a tracing of the resulting TLC plate, spotted and developed for three hours as described in the Experimental section. Toxins are identified on the profile by number.

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Application of a mixture of group B toxins to carboxylate resin in the ammonium form resulted in sorption of toxins 2, 3, and 5, while 8, 9, and 11 were eluted by pH 7.5 ammonium acetate buffer. This is presumed to reflect deprotonation of the N-1-hydroxyl in 8, 9, and 11, rendering them uncharged.

Binding of applied material to carboxylate resins in the hydrogen form liberates protons which, depending on the composition of the applied sample, lowers the *in situ* pH. The bulk concentration of carboxylate in the resin bed is about 3 M, so the effect can be substantial. During the water wash following sample application, effluent pH and conductivity showed a spike corresponding to unretained material with a pH often below 2. This effect interferes with toxin binding, particularly when the sample has a large component of exchangeable cations and strong acid anions, and particularly with the group B toxins. Two strategies were employed to circumvent this. First, relatively pure toxin mixtures were applied as acetates in solutions dilute enough that the resulting *in situ* hydrogen ion concentration was low enough to permit binding. Second, application of more concentrated samples was preceeded by application of a few millimoles of ammonium hydroxide and a brief water wash. This was found to insure retention of all the group A and B toxins, presumably by moderating *in situ* pH although, in the case of 8, 9, and 11, sorption may have depended on a transition zone between that of high pH in the section of ammonium-carboxylate bed and low pH in the main H-carboxylate bed.

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II.B.3c. Sephadex G-10

Isocratic elution of group B mixtures from Sephadex G-10 with 0.1 M acetic acid was the only system found useful for separating 2 and 8 from 3, 5, 9, and 11, the sulfamates 2 and 8 eluting just ahead of the other four. Resolution was incomplete, 8 in particular trailing back into the unresolved cluster of 3, 5, 9, and 11.

II.B.3d. Thin Layer Chromatography

Toxins in chromatographic effluents were routinely located and identified using the spot test and TLC methods developed by Buckley (Buckley *et al.*, 1976).

In the spot test, aliquots from fractions are applied as a series of spots to a strip of TLC plate, dried, sprayed with hydrogen peroxide, and heated briefly. Under these conditions all the toxins oxidize to form derivatives fluorescent under 366 nm UV light. Substances that are not fluorescent before, but become fluorescent as a result of this treatment are designated 'Buckley spot positive'. The test is sensitive and selective for saxitoxins (Shoptaugh *et al.*, 1978). In the present work, no evidence was found for acute, PSP-like toxicity that was not due to Buckley spot positive substances.

The fluorescence of spots resulting from toxins 1-6 tends to appear blue, while that from toxins 7-12 tends to appear yellowish. The appearance depends somewhat on conditions of preparation and so must be interpreted with caution, but is a consistent trend for each toxin and therefore permits useful inference regarding N-1-hydroxy substitution.

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Given the power of this test, it is interesting to note that the formation of fluorescence by preparations of shellfish toxin was observed as early as the work of Bendien and Sommer (1941), but the analytical implications were not exploited until Bates and Rapoport (1975) developed a fluorometric assay for saxitoxin using hydrogen peroxide in basic solutions. A related method was developed for the continuous monitoring of chromatographic effluents (Buckley *et al.*, 1978). Both methods, however, suffer from the very low yields of fluorescent products obtained in solution from the N-1-hydroxy toxins, in contrast to the uniformly high yields obtained in this and other studies for spots of both the N-1-H and N-1-OH toxins on silica gel TLC plates.

TLC on silica gel plates using the solvent system developed by Buckley and slight modifications of it, such as that used here (Shimizu, 1978a), remains the most powerful TLC method for resolution of the toxins. Visualization of the toxins on developed plates is again by oxidation to fluorescent products. Relative migration position of the various toxins was found to be highly consistent and, coupled with elution volume from column chromatography, led to two-dimensional arrays with coordinates characteristic of each toxin (Figures 13, 14). Numerical R_f values are not quoted because they were found to vary slightly, particularly with repeated use of a batch of solvent, and can be misleading.

The Buckley TLC system is limited in that it requires relatively pure material and does not resolve all the toxins. The utility of the BioGel P2 column method developed in the present study is that, although

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it does not achieve resolution of all twelve toxins, it removes the bulk of non-toxic material that otherwise interferes with TLC and resolves the toxins that overlap on TLC.

Although the plates were routinely scanned for substances that were fluorescent or UV absorbing (366 and 254 nm), substances that were neither and were not Buckley spot positive would have gone undetected. On the other hand, there was at least one component, eluting from Bio-Gel well ahead of saxitoxin and observed in most extracts, that was Buckley spot positive and devoid of toxicity before or after heating with HCl. The existence of such a component suggests that using the fluorescence of extracts following peroxide oxidation (Bates and Rapoport, 1975) as an index of toxicity could be misleading.

II.B.4. Preparation of Pure Toxins

Since the understanding of toxin chemistry evolved as the work was being done, there was no case in which purifications were carried out starting with raw extract and ending up with pure material by a sequence that would, in hindsight, be considered rational. The following reflects the concepts that developed. Figure 16 outlines a reasonable sequence.

Chemical studies were conducted with extracts of clone PI07, which was later found not to contain $\underline{1}$, $\underline{3}$, or $\underline{5}$. Substantial amounts of these, however, were obtained from the inadvertent hydrolysis of $\underline{2}$, $\underline{4}$, and $\underline{6}$, before the nature and sensitivity of the 21-sulfo toxins were recognized. Extraction with dilute acetic acid at room temperature was found

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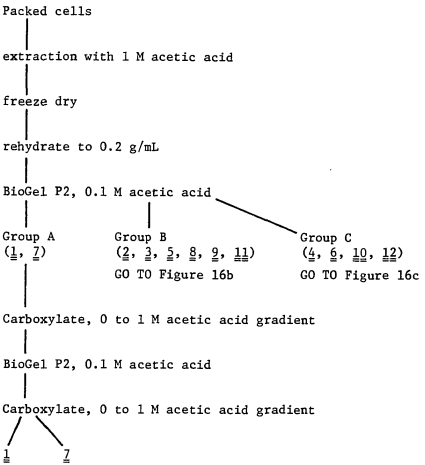

Figure 16a. Preparative separation of the saxitoxins. Preliminary and group A.

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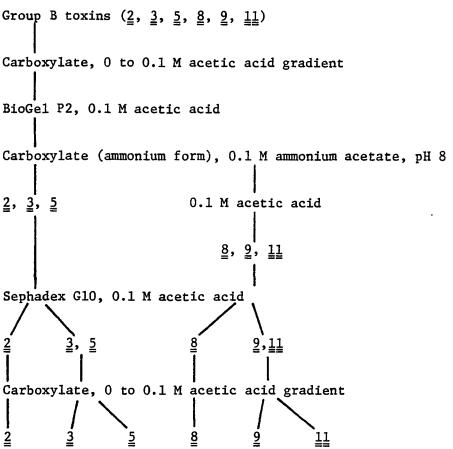

Figure 16b. Preparative separations of the saxitoxins. Group B.

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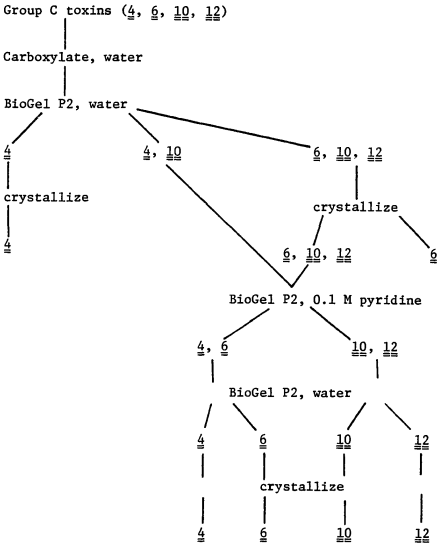

Figure 16c. Preparative separation of the saxitoxins. Group C.

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satisfactory. Hydrochloric acid may be used provided that the amount of acid is small compared to the buffer capacity of the cell matter present, or a buffer such as ammonium acetate is added prior to drying.

Initial separation of the freeze-dried extracts is best accomplished by isocratic elution from BioGel P2 with 0.1 M acetic acid, resolving the toxins from the bulk of non-toxic material and into the groups A, B, and C.

Resolution of the group A toxins $\underline{1}$ and $\underline{7}$ on carboxylate resins was slightly better than that attained on BioGel P2.

Separation of the group B 21-sulfo toxins $\underline{2}$ and $\underline{8}$ from the 11-hydroxysulfates $\underline{3}$, $\underline{5}$, $\underline{9}$, and $\underline{11}$ on Sephadex G-10, or of the N-1-OH toxins $\underline{8}$, $\underline{9}$, and $\underline{11}$ from the N-1-H toxins $\underline{2}$, $\underline{3}$, and $\underline{5}$ on ammonium-carboxylate resins can be performed in either order. However, the ammonium-carboxylate step is most convenient when performed with a small bed volume (1-2 mL), simplifying the recovery of the bound toxins $\underline{2}$, $\underline{3}$, and $\underline{5}$.

Freeze-drying of solutions containing traces of strong acid in excess of the buffer capacity of the system was found to result in hydrolysis of the 21-sulfo toxins. The problem was particularly acute with the group C toxins which, lacking the acetate counterion, have no buffer capacity. The composition of the group C toxins furthermore makes them potentially autolytic, since hydrolysis of the 21-sulfo group results in 1 equivalent excess strong acid. A small amount of pyridine or ammonium acetate was therefore routinely added to solutions of the 21-sulfo toxins prior to freeze drying except in the final stages of purification.

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Because of their facile epimerization, resolution of the four pairs of 11-hydroxysulfates is best reserved for later steps in the sequence. Isocratic elution from BioGel P2 with 0.1 M acetic acid or gradient elution from H-carboxylate resin each provided fair resolution of the epimeric pairs 3,5 and 9,11. Elution from BioGel P2 with water achieved nearly baseline resolution of the pairs 4,6 and 10,12.

Passage of crude group C toxins through a short column of H-carboxylate resin was useful for removing pigmented material and traces of other toxins, but was treacherous in that the toxins emerge along with other unretained material which is depleted in non-proton cations and includes strong acid anions. It is essential that the group C toxin solution be adequately buffered following this step.

Quite unexpectedly, toxins 4, 6, 10, and 12 were found to crystallize. The solubility of 6 in water is about 50 mg/mL at 50°C and 10 mg/mL at 0°C, the compound being obtained as large crystals from hot water (Figures 17, 18). Compound 4 proved quite soluble in water, over 100 mg/mL at room temperature, but was obtained as small prisms from methanol/water mixtures (Figure 19). The mother liquors from crystallizations of 4 and 6 are sufficiently enriched in 10 and 12 that they can be easily detected and resolved from 4 and 6 by chromatography on BioGel P2 at pH 8, then resolved from each other and crystallized by methods corresponding to those used for 4 and 6.

With the exception of a sample of 5, prepared as the hydrochloride for ^{13}C-NMR, final purification of individual group A and B toxins involved binding to H-carboxylate resins and elution with acetic acid, to

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Figure 17. A crystal of toxin C2 (6). Bar is 1 mm.

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Figure 18. Crystals of toxin C2 (6) that have lost crystallinity following exposure to room temperature dry air for a few minutes. Bar is 1 mm.

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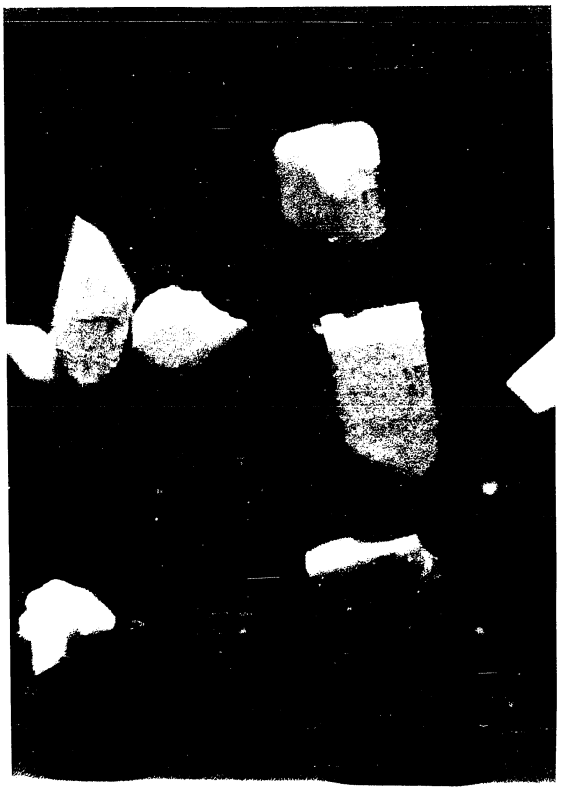

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Figure 19. Crystals of toxin C1 (4). Bar is 1 mm.

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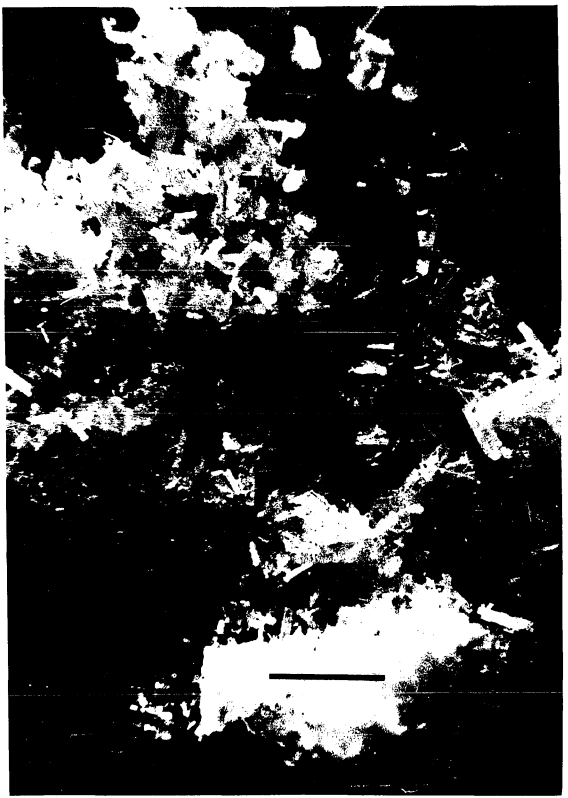

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give maximum assurance that the counterion was acetate. Combustion analysis of 8 suggested that this strategy was successful, and that it is therefore permissible to assign formula weights for the preparations, allowing the potencies of the toxins to be expressed on a molar basis (section II.B.5) and standards of prescribed molar concentration to be formulated (Figure 14). Since combustion analyses were not obtained for the majority of toxins, the degree of hydration is uncertain except for 6 and 8 (section II.B.6c). Each equivalent of water, however, would alter the formula weight by less than 5%, so the error was likely insignificant in the present work.

II.B.5. Potencies of the Toxins

The potencies of eight of the *Protogonyaulax* neurotoxins were determined by mouse bioassay and are summarized in Figure 20. Assays were performed both before and after heating with HCl, in most cases on several distinct preparations of each toxin. Uncertainty with respect to the degree of hydration, less than 5% per equivalent of water that might be bound, is unlikely to have caused significant error.

It is reassuring to note the close agreement between the potencies of the 21-H toxins and those of the corresponding 21-sulfo toxins following hydrolysis of the 21-sulfo group. This argues for the purity of both members in each pair, since they differ in properties and were therefore purified by different routes.

The recently published values from Genenah and Shimizu (1981) are included in Figure 20 for comparison. In their study, the molar

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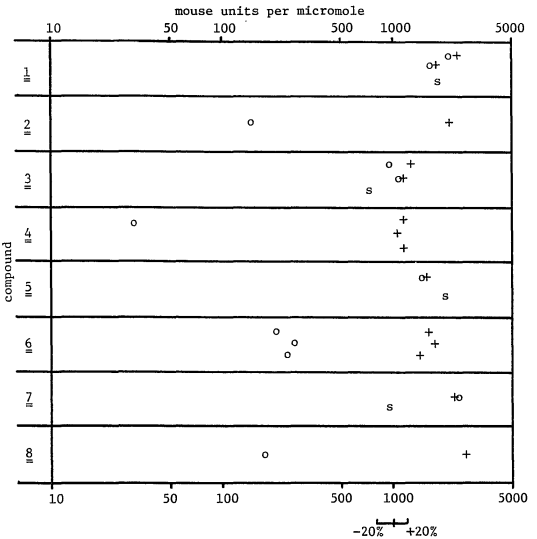

o = potency of the raw solution
+ = potency of the solution following hydrolysis (ca. 0.2 M HCl, 100°C, 5 minutes)
s = values for comparison, from Genenah and Shimizu (1981)

Figure 20. Potencies of several *Protogonyaulax* neurotoxins. A log scale is used to better accommodate the range of values and to simplify visual comparison of dispersion with the uncertainty of the mouse bioassay.

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concentration of toxin was determined by nitrogen analysis of the dried solution, assuming seven atoms of nitrogen per molecule of toxin and neatly circumventing uncertainties about hydration. Their value for 1 is in good agreement, but their value for 7 is far too low, suggesting that their preparation was impure. The same might be said for 3 were it not for the difference seen in 5. Taken together, the discrepancies could be explained if all the preparations of 3, 4, 5, and 6 assayed in the present work had partly epimerized, approaching therefore a median value for the equilibrium mixture of 11-hydroxysulfate epimers. Since the samples of 4 and 6 were prepared directly from crystalline material, this seems unlikely. The question requires further attention before it can be resolved.

II.B.6. Structure Elucidation of the 21-Sulfo Toxins

II.B.6a. Transformations

The conversions responsible for Proctor enhancement were evaluated by TLC of solutions before and after heating with dilute HCl, with the following results:

	composition:					
before treatment	2	4	6	8	10	12
after treatment	1	3	5	7	9	10

Toxins 4 and 6 were found to interconvert, 4 predominating in equilibrated mixtures. Interconversion was accelerated by warming and by the presence of ammonium or pyridinium acetate. Toxins 10 and 12 were

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similarly found to interconvert, 10 predominating in equilibrated mixtures.

II.B.6b. Net Charge

In acidic solutions, 2 and 8 appeared to bear a net charge of +1 on the basis of their chromatographic affiliation with 3, 5, 9, and 11, while the non-retention of the group C toxins 4, 6, 10 and 12 indicated that they had no net charge.

Paper electrophoresis of some of the toxins at pH 4.5 was performed by Frank Koehn at the University of Wisconsin and gave the following mobilities relative to saxitoxin = 1.00:

Compound:	3	4	5	6	7	8
Mobility:	0.62	0.00	0.60	0.00	0.93	0.62.

This confirms the absence of net charge in 4 and 6 and suggests that the charge of 8 differed from 7 by the same increment that exists between 1 and its hydroxysulfates 3 and 5.

Taken together with the hydrolyses and interconversions, the above indicate that the toxins 2, 4, 6, 8, 10, and 12 differ from 1, 3, 5, 7, 9, and 11 by the presence of a substituent charged -1.

II.B.6c. The Substituent

Combustion analyses of 2 and 6, dried on the freeze dryer at about 10 microns, gave the following results:

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For 8 acetate, $C_{12}H_{21}N_7O_{10}S$:

	C	H	N	O	S
calculated:	31.65	4.65	21.53	35.13	7.04
found:	31.41	4.60	22.33	35.17	6.58

For 6 monohydrate, $C_{10}H_{19}N_7O_{12}S_2$:

	C	H	N	O	S
calculated:	24.34	3.88	19.87	38.91	13.00
found:	24.39	3.88	19.78	38.59	12.89

The results show that the substituent in toxins 8, 6, and, by implication, 4, to be an $-SO_3$ group. It is interesting to note that under the drying conditions used 6 retains one equivalent of water of hydration.

II.B.6d. NMR Spectra

NMR spectra of the purified toxins were obtained through collaboration with Dr. Heinrich K. Schnoes at the Department of Biochemistry, University of Wisconsin. The spectra were run by Carol Fix Wichmann. Table 4 summarizes the ^{1}H-NMR data, Table 5 the ^{13}C-NMR data. Proton chemical shift differentials between the carbamate and sulfamate toxins are charted in Figure 21.

Data for most of the carbamate toxins are from previous work by the Wisconsin group. Toxin 5 had not previously been available in quantities sufficient for ^{13}C-NMR spectra, however, and was supplied from this work. For this it was necessary to use the hydrochloride, rather than the

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Table 4. ^{1}N-NMR data.[1,2]

Compound	Proton						
	H-5	H-6	H-10	H-10	H-11	H-13	H-13
1	4.33 d (1.3)	3.47 ddd (1.3,6,9)	3.20 ddd (8,10,11)	3.45 ddd (2.5,10,11)	2.00 m	3.65 dd (6,12)	3.88 dd (9,12)
2	4.34 d (1.3)	3.48 ddd (1.3,6,9.5)	3.20 m [3]	3.40 m [3]	2.00 m	2.72 dd (6,13)	4.00 dd (9.5,12)
3	4.41 d (1.0)	3.46 ddd (1.0,5.6,9.0)	3.60 dd (5.0,12.7)	3.76 d (12.1)	4.44 d (4.8)	3.64 dd (5.4,11.9)	3.85 dd (9.3,11.2)
4	4.42 s	3.50 dd (5.3,9.5)	3.63 dd (4.8,12.2)	3.77 d (12.2)	4.43 d (5.0)	3.76 dd (5.5,11.8)	3.96 dd (9.8,11.8)
5	4.44 d (1.0)	3.41 dd (1.0,6.0,9.0)	3.18 dd (7.0,11.0)	3.75 dd (8.8,11.0)	4.56 dd (7.5,8.5)	3.67 dd (5.5,12.0)	3.87 dd (9.0,11.8)
6	4.45 d (1.0)	3.51 ddd (1.0,5.4,9.3)	3.23 dd (7.0,11.0)	3.79 dd (7.5,10.8)	4.58 dd (7.6,7.0)	3.81 dd (5.0,11.9)	3.99 dd (9.5,11.8)
7	4.42 d (1.1)	3.70 ddd (1.1,6,6.2)	3.18 ddd (7,10.2,9.7)	3.38 ddd (2.7,10.2,10)	1.98 m	3.82 dd (6.2,11.8)	4.00 dd (5.9,11.8)
8	4.43 d (1.1)	3.76 dt (1.1,6.8)	3.24 m [3]	3.39 m [3]	1.95 m	3.88 dd (6.8,12)	4.14 dd (6.8,12)
9	4.50 s	3.70 dd (5.4,6)	3.57 dd (4.9,11.6)	3.75 d (11.3)	4.45 d (4.9)	3.83 dd (5.9,12.3)	4.01 dd (5.8,11.8)
10	4.52 s	~3.8 m	3.64 dd (4.8,12.5)	3.74 d (12.0)	4.47 d (4.5)	3.87 dd (6.0,12.0)	4.13 dd (6.5,12.0)
11	4.54 s	3.72 dd (6.5,6)	3.21 dd (7.4,10)	3.78 dd (7.5,10.4)	4.57 dd (7.5,7.4)	3.89 dd (5.9,12.2)	4.04 dd (6,12.6)
12	4.54 s	[3.78][4]	3.24 dd (9.5,10)	[3.78][4]	4.59 t (7.5)	3.93 dd (6.8,11.8)	4.14 dd (6.2,11.2)

Table 4. Continued.

[1]Chemical shifts in ppm with reference to $CHCl_3$ at δ = 7.27 as internal standard. Data in () are coupling constants in Hz.

[2]Data for the carbamate toxins (1, 3, 5, 7, 9, and 11) are primarily from earlier work by the Wisconsin group.

[3]Reliable determination of the H-10 coupling constants for 2 and 8 was precluded by partial deuterium exchange of the H-11, catalyzed by acetate counterion. Fully exchanged samples showed an H-10 geminal coupling constant of 11 Hz for 2 and 8.

[4]Assignments uncertain.

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Table 5. ^{13}C-NMR data.[1,2]

compound	Carbon 2	4	5	6	8	10	11	12	13	19
1	156.6	83.0	57.5	53.6	158.4	43.5	33.5	99.1	63.8	159.3
2	154.8	83.1	57.6	53.4	156.7	43.2	[3]	99.1	64.2	158.8
3	158.0	81.6	57.9	53.2	156.2	51.1	77.7	97.6	63.3	159.0
4	156.8	82.1	58.4	53.5	154.6	51.5	78.1	98.0	64.5	158.7
5	158.6	82.5	58.1	53.8	156.4	48.3	76.6	98.1	63.9	159.6
6	156.5	82.6	58.1	53.7	154.7	48.3	76.6	98.2	64.6	158.8
7	159.1	82.6	57.4	61.6	158.6	44.3	33.8	99.2	64.8	159.3
8	158.8	82.1	57.1	62.0	154.3	44.1	33.7	99.1	67.6	158.9

[1] Shifts are in ppm from TMS with dioxane at δ = 67.6 as internal standard.

[2] Data for compounds 1, 3, and 7 are from earlier work by the Wisconsin group.

[3] Resonance for carbon 11 in compound 2 was not observed due to deuterium exchange of the carbon 11 protons.

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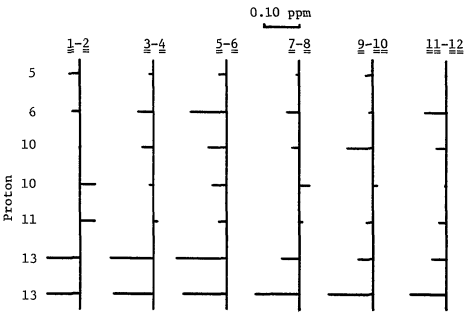

Figure 21. Proton chemical shift differentials between respective pairs of carbamate and sulfamate toxins. Vectors show the shift of each peak in the sulfamate toxin from the corresponding carbamate toxin.

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acetate, since acetate-catalyzed epimerization would have been excessive over the period of the run.

Both the proton and carbon spectra of 2 and 8 were run on the acetates. In the carbon spectrum of 2, the 11 carbon was not observed because the sample was shipped in D_2O solution and the C-11 protons had exchanged. This was avoided in the case of 8 by shipping the sample dry.

Assignments for the sp^2 region of the carbon spectra are discussed in section II.B.6i. Assignment of the remaining peaks in the carbon spectra and in the proton spectra of the carbamate toxins are based on work by the Wisconsin group. The spectra of the sulfamate toxins differ so little from the corresponding carbamates that most assignments can be made with reasonable certainty.

II.B.6e. Structural Hypothesis

As it became clear that the new toxins differed from those to which they hydrolyzed only by the presence of an $-SO_3$ group, the question became where to place it. While retrospective evaluation of the sp^2 region of the ^{13}C-NMR spectra suggests that, properly interpreted, they suffice to place the substituent on the carbamoyl group and proton shift differentials pointed to alteration somewhere on the side chain, the first rational hypothesis for the position of the substituent came from ^1H-NMR spectra of 3, 4, 5, and 6 prepared in d_6-dimethyl sulfoxide by Carol Fix Wichmann and interpreted by Dr. H. K. Schnoes. In compounds 5 and 6, peaks are observed for all heteroatom-bonded protons, including in 5 a two-proton singlet at $\delta = 6.58$ which, in 6, is missing but

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replaced by a one proton singlet at δ = 9.37. Although similar spectra of 3 and 4 are less clear, corresponding peaks are observed in 3 at δ = 6.61 (two proton singlet) and in 4 in δ = 9.28 (one proton singlet). The two proton signals in 3 and 5 were assigned to the protons on the carbamate nitrogen, one of which is replaced in 4 and 6 by the sulfo group, shifting the resonance of the remaining proton.

II.B.6f. Structures of C1 and C2

X-ray diffraction studies of crystalline material, performed at the University of Akron by Dr. Stephen D. Darling, led to the structure for C2 (6) shown in Figure 22. These studies confirmed not only the 21-sulfo substitution, but also that the previous assignments of the stereochemistry at C-11, based on spectroscopic inference (Boyer, 1980) were correct.

Rotation of the structure to align the bond between carbons 6 and 13 (Figure 23) reveals that the orientation around the bond corresponds to that in crystalline saxitoxin ethyl hemiketal (20), as discussed by Shimizu *et al.* (1981).

The unit cell contains four molecules of 6. The structure was refined to a final R = 0.199, a relatively high uncertainty in refinement.

Although crystals of 6 were easy to produce and were of excellent size and quality, they 'chalked' on exposure to air at room temperature (Figure 18). They appeared indefinitely stable when stored in mother liquor or in a moist atmosphere. Diffraction studies were performed on crystals mounted in capillaries. Determinations of the weight loss on

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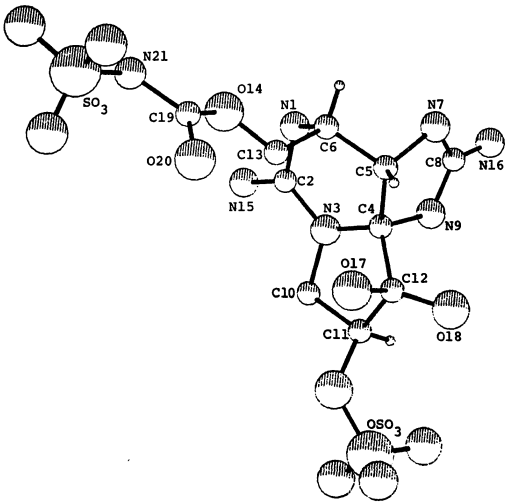

Figure 22. X-ray crystal structure of toxin C2 (6), obtained by S. D. Darling at the University of Akron. The positions of protons on carbons 5, 6, and 11 were calculated and are included for stereochemical clarity.

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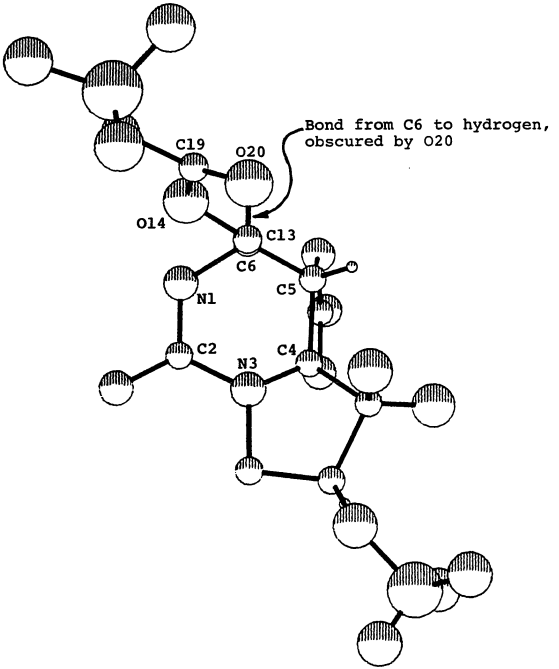

Figure 23. Structure of toxin C2 (6), rotated to clarify the orientation of substituents around carbons 6 and 13.

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drying, coupled with combustion analysis of the dried material, indicate that each unit cell contains approximately 16 molecules of water that have not yet been accounted for in the x-ray structure and may help explain the relatively high R value.

Crystallizations of 4 under a variety of conditions gave crystals of several different morphologies, most rather pretty, but none suitable for structure determination. However, the assigned structure seems secure, based on 1) the reversible interconversion of 4 and 6; 2) the hydrolysis of 4 to 3; 3) ^{13}C-NMR; and 4) ^{1}H-NMR in both D_2O and DMSO.

II.B.6g. Structures of B1 and B2

Given the structures of 4 and 6, the probable structures of 2 and 8 are immediately apparent on the basis of their hydrolyses to 1 and 7 respectively, their ^{1}H-NMR and ^{13}C-NMR spectra in D_2O, and their chromatographic behavior. However, efforts in Seward to crystallize 2 or 8 were unproductive, as were attempts by the Wisconsin group to obtain interpretable ^{1}H-NMR spectra in DMSO. Success with either technique might have sufficed to confirm the assignments. Formal confirmation of the tentative structural assignments of B1 as 2 and B2 as 8 came instead from the synthesis, by Frank Koehn at the University of Wisconsin, of each from the corresponding decarbamoyl toxins 15 and 16.

In the total synthesis of d,l-saxitoxin (Tanino *et al.*, 1977), chlorosulfonyl isocyanate (Graf, 1963) had been used to place the carbamate group on oxygen-14. Remarks by Graf (1963) in his original work with the reagent suggest the N-sulfo group as an intermediate in the

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formation of derivatives. Koehn found that quenching a reaction mixture containing chlorosulfonyl isocyanate and either 15 or 16 with cold ammonium acetate indeed permitted recovery of the intermediates 2 or 8, respectively, in good yield (Koehn *et al.*, 1982).

II.B.6h. Structures of C3 and C4

Compounds C3 (10) and C4 (12) were at first notable by their absence. Finding toxins 2, 4, 6, and 8, the 21-sulfo derivatives of 1, 3, 5, and 7, strongly suggested that there might be corresponding derivatives of 9 and 11, but C3 and C4 were not recognized in the initial chromatographic studies (Hall *et al.*, 1980) being present at relatively low concentrations. Finally, in the mother liquors resulting from crystallizations to obtain 4 and 6, C3 and C4 became sufficiently enriched for detection. Again, chromatographic behavior, hydrolysis to 9 and 11, interconversion of C3 and C4, and ^{1}H-NMR data made it obvious (while failing to formally prove) that C3 and C4 were the expected compounds 10 and 12. It was, however, also expected that C3 and C4 could be crystallized, on the basis of experience with 4 and 6. Unambiguous determination of the structures was of particular importance because it would provide the first independent confirmation for the structure of neosaxitoxin, an assignment made on the basis of elegant, though tenuous, spectroscopic arguments (Shimizu *et al.*, 1978b). The structures of the sulfamates would also serve to confirm those of the corresponding carbamates 9 and 11.

Given compounds C3 and C4, purified by chromatography, it was indeed found that they crystallized using methods and in forms corresponding

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to those for 4 and 6, respectively. Compound C3 was relatively soluble in water, but formed small prisms from ethanol/water mixtures. Compound C4 had a relatively low water solubility and formed large crystals, suitable for x-ray work, on the evaporation of a water solution. Some of these were mounted in capillaries and mailed to the University of Akron, where x-ray diffraction studies by Dr. S. D. Darling eventually provided the structure shown in Figure 24. Note the N-1-hydroxyl, 21-sulfo, and orientation of the 11-hydroxysulfate, confirming in all respects the expected structure 12 and, by implication, those of compounds 7, 8, 9, 10, and 11. The unit cell parameters do not differ significantly from those for 6. At present, only preliminary refinement has been completed but, after two iterations, R has condensed to 0.136 suggesting that, in contrast to the experience with 6, a precise refinement can soon be obtained.

II.B.6i. Comments on the N-Sulfocarbamoyl Group

While the carbamoyl-N-sulfo group appears to be without precedent among natural compounds, Graf (1963) alluded to such a structure as an intermediate stage in the formation of derivatives using chlorosulfonyl isocyanate. Nitrogen-sulfur bonds in general appear to be relatively uncommon in nature (Davis and Morris, 1981), although some interesting examples exist. The most relevant are sulfazecin (Imada *et al.*, 1981) and other recently discovered 'monobactam' antibiotics (Sykes *et al.*, 1981), obtained from strains of *Pseudomonas* and other bacteria. The monobactams share the novel beta lactam ring 26.

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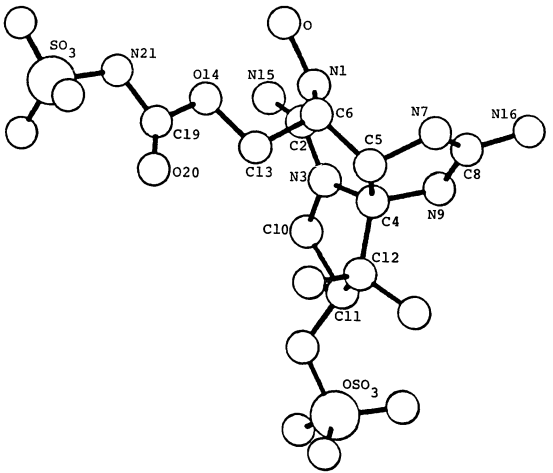

Figure 24. X-ray crystal structure of toxin C4 (12), obtained by S. D. Darling at the University of Akron.

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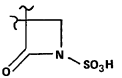

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The N-S bond of the monobactams appears to be more stable to hydrolysis than that of the sulfamate toxins. Thus, hydrolysis of sulfazecin at 37°C in 1 M HCl cleaves the ring but leaves the N-S bond intact (Imada *et al.*, 1981). It is tempting to speculate that the lack of previous reports of carbamoyl-N-sulfo compounds is due in part to their facile hydrolysis.

As mentioned in section II.B.6d, assignments in the sp^2 region of the ^{13}C-NMR spectra (Figure 25) of the toxins are not entirely clear. Those for saxitoxin (1) taken from Koehn *et al.* (1981), are C-19, 159.7; C-8, 158.5; and C-2, 156.8. Assignment in the same sequence appears reasonable for compounds 3 and 5. In compound 7, the three peaks form a tight cluster, making assignments uncertain. The change is probably due to a downfield shift of the signal for carbon 2, so those shown for 7 in Table 5 seem likely.

While the changes in the signals for carbons 2, 8, and 19 seen in the sulfamate toxins 2, 4, 6, and 8 could be interpreted as an upfield shift of all three it seems more likely, given the uniform appearance of one peak at about 154.5 ppm, that instead the carbonyl resonance is shifted from about 159.5 ppm in the carbamate, upfield to about 154.5 in the sulfamate. This corresponds well to the 3.4 to 4.7 ppm upfield shift for the amide carbonyl observed in the sulfonation of primary amides (Floyd *et al.*, 1981) during synthesis of the monobactams. It is

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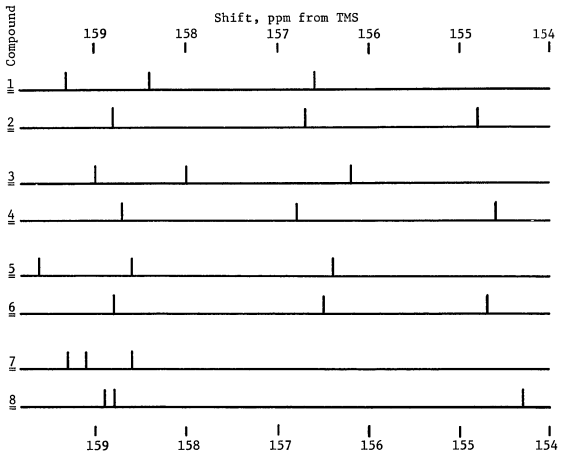

Figure 25. ^{13}C-NMR shifts in the sp^2 region for several *Protogonyaulax* neurotoxins. Note the resonance between 154 and 155 ppm observed for the sulfamate toxins 2, 4, 6, and 8.

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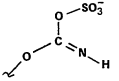
likely that the question can best be resolved by the introduction of ^{13}C label at C-19, using the methods of Koehn *et al.* (1982).

While hydrolysis of the monobactam ring conserving the N-S bond clearly demonstrates N-sulfonation in the cyclic compounds, it should be noted that neither the crystal structures of 6 and 12 nor available spectroscopic evidence can completely exclude the isomeric form (27)



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as an alternative for the solution structure of the sulfamate toxins.

II.B.7. Other Toxins

The toxin array presented herein appears to represent the natural assembly extractable from *Protogonyaulax* and suggests that modifications of the saxitoxin skeleton other than N-1-hydroxy, 11-hydroxysulfate, and 21-sulfo do not occur. However, other modifications are well known *in vitro* (such as those discussed by Koehn *et al.*, 1981) and several other toxins, the structures of which have not been reported, have been mentioned in the literature. These appear related to saxitoxin, being Buckley spot positive and causing similar responses in bioassays.

Gonyautoxin 5 has been reported from the majority of shellfish and *Gonyaulax* samples analyzed by Shimizu and his associates (Shimizu, 1979), while gonyautoxin 6 has been reported only from shellfish. Available chromatographic data, while not entirely in agreement, suggest that they correspond to 2 and 8, respectively. Both GTX 5 and GTX 6 were reported

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in analyses of mussels from Haines and Elfin Cove (Shimizu, 1978a), *Protogonyaulax* from both locations being found in the present study to contain 2 and 8 (see Table 10).

Gonyautoxin 8 (Kobayashi and Shimizu, 1981) appears to correspond to C2 (6) on the basis of NMR spectra, toxicity, and chemical properties.

Gonyautoxin 7 (Hsu *et al.*, 1979) has been reported only from Bay of Fundy scallops and apparently corresponds to an unknown toxin reported by Fix Wichmann *et al.* (1981a), also from Bay of Fundy scallops. Data from the two reports suggest that the substance is a dication related to 1, distinct from 7 and 15, probably lacking an N-1-hydroxyl. Turban shell toxin (Kotaki *et al.*, 1981) from tropical gastropods of the genera *Tectus* and *Turbo*, appears to be yet another dication related to 1, distinct from 7, 15, and GTX 7.

Accompanying 1 in the blue green alga *Aphanazomenon flos-aquae*, Alam *et al.* (1978) found three toxins apparently related to 1 but chromatographically distinct from known saxitoxin derivatives.

In the present study, an extract that had been exposed to a variety of abusive conditions was found to contain two group B toxins, separable from all others, that apparently had no N-1-hydroxyl (blue Buckley spot) and no 21-sulfo (no alteration with heating in 0.2 M HCl). They interconvert and, given their chromatographic properties, seem likely to be 11-hydroxysulfate epimers without other charged substituents on a saxitoxin nucleus. One of them was purified to about 1,000 mouse units per milligram. The above properties would identify them as 3 and 5 from which, however, they are chromatographically distinct. Since no trace

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of them was detected in routine analyses of *Protogonyaulax* extracts, it would appear that they were formed as artifacts during manipulation of the extract. It is conceivable that they are decarbomoyl 11-hydroxy-saxitoxin sulfates $\underline{\underline{23}}$ and $\underline{\underline{24}}$.

Other trace spots were occasionally noted in the chromatography of extracts. From their low intensities it would appear that, even if they are toxins, they are quantitatively insignificant. This is a weak criterion, however, since toxins $\underline{\underline{10}}$ and $\underline{\underline{12}}$ were similarly observed only at very low intensity, but are essential to the concept of the complete array.

The overwhelming majority of mice injected with *Protogonyaulax* extracts or fractions either died promptly with symptoms characteristic of the saxitoxins or survived without apparent ill effect. This supports the idea that toxicity in *Protogonyaulax* is due essentially to the saxitoxins. However, some fractions lacking in apparent saxitoxin-like activity were noted to cause unusual distress in mice during injection. The mice subsequently were quiescent, obviously not well, survived several hours at least, and died unobserved during the following day or two. Consistent with the early reports by Sommer (Sommer *et al.*, 1937), it appears likely that relatively low levels of toxins with different effects accompany the more apparent saxitoxins in these organisms.

II.B.8. Analytical Methodology

II.B.8a. Introduction

Isocratic elution from BioGel P2 coupled with TLC to give profiles such as that shown in Figure 13, proved to be a convenient and effective

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method for analyzing toxin composition in *Protogonyaulax* extracts and was used for the survey presented in sections II.C.2 and II.C.3. This section outlines the method and describes studies to refine and evaluate it.

II.B.8b. Outline of the Method

For analysis, cells were harvested by pouring the culture through a plankton net, the resulting concentrated cell suspension centrifuged, and the packed cells extracted. The extract was freeze-dried, resuspended in water, and a portion applied to the column. Fractions were collected and evaluated using the Buckley spot test and TLC of spot-positive fractions. On the basis of TLC, similar fractions were pooled for assay. In Tables 6, 8, and 9, these results were summed for group A, B, and C toxins, for all toxicity eluting before group A ('early'), and for that eluting about a bed volume after group C ('late'). In addition to assays of the applied suspension and the original extract, both the seawater supernatant from centrifuging the harvested cells and the cell pulp following extraction were assayed. In the later case, the cell pulp was digested with HCl, the pH adjusted, and the assays performed without further treatment. Aliquots of the other solutions were heated with HCl, so that all assays reflect toxicity following Proctor enhancement. Toxicities are expressed on the basis of micromouseunits/cell in the original sample. In the qualitative entries, $\underline{\underline{10}}$ and $\underline{\underline{12}}$ are omitted since they were generally undetectable in routine analyses. Entries for the other 11-hydroxysulfates are paired since in every case

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the β epimer predominated and was accompanied by small amounts of the α epimer. The notations 3/5, 4/6, and 9/11 are used for these pairs.

II.B.8c. Methods for Sample Preparation

The design of preparation methods was based primarily on two series of replicate samples taken on two occasions from the same vat culture of clone PI07. The results are summarized in Table 6. Evaluation is based on these and the results shown in Tables 8 and 9. Three questions were considered: 1) loss of toxins from the cells during harvest; 2) recovery of the toxins from cells by extraction; and 3) alterations of the toxins by extraction.

Loss during harvest: Some amount of toxin would be expected to leak from cells in their normal state. Prakash (1967) detected some toxicity in the medium of older cultures of *Gonyaulax tamarensis*. However, due to the gross levels of toxicity found in cultures, a few mouse units per milliliter being the upper limit, and to the amount of salt in the seawater medium, toxin leaked into the medium is difficult to assay or analyze. While the amount of leakage to the medium that might be induced by the initial filtration through the plankton net is therefore difficult to directly assess, some estimate of a lower limit of such loss is provided by assays of the seawater supernatant that resulted from centrifuging the cell concentrate thus obtained.

The results range from about 2% to an exceptional value of over 30% of the recovered toxicity leaking into the seawater supernatant, about 10% being typical. The losses tended to be smaller, although not

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Table 6. Variation of toxin composition with conditions of sample preparation.

	Extraction[1]			Chromatography[1]									
	Seawater supernatant	Pulp	Extract	Applied suspension	Early	A	B	C	Late	Composition 1 ≙	2 ≙	3/5 ≙	4/6 ≙	7 ≙	8 ≙	9/11 ≙
0.1 M aqueous acetic acid																
	25[4]	22	230							+			+	+	+	+
0.03 M aqueous HCl, buffered with ammonium acetate before drying																
	20[4]	5	230							+			+	+	+	+
0.1 M aqueous acetic acid, frozen and thawed																
	7[5]	36								+			+	+	+	+
1 M aqueous acetic acid																
	9[5]	4	370	340	6	32	120	110	8	+			+	+	+	+
boiled with 80% ethanol, 0.01 M in HCl																
	7[5]	7		310	13	110[3]	46[3]	2	9	+		+		+		+[2]
0.05 M aqueous HCl																
	8[6]	4		150	18	140	29	2	2	+				+		
methanol, 0.5 M in acetic acid																
	17[6]	94								+			+	+	+	+
80% ethanol, 0.5 M in acetic acid																
	39[6]	31		200	6	24	97	71	9	+			+	+	+	+

[1] Values are toxicities in micromouseunits/cell which, due to the imprecision of the mouse bioassay, have an uncertainty on the order of ±20%.

[2] Weak.

[3] Fractions include low R_f spots, possibly 11-hydroxysaxitoxins.

[4] Cells removed from seawater suspension by centrifuging 20 minutes at 1500 g.

[5] Cells centrifuged from seawater suspension by accelerating to 1500 g in 30 seconds and decelerating over 3 minutes.

[6] Cells centrifuged from seawater suspension at 500 g for 5 minutes.

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consistently so, if centrifugation was brief. There is no present basis for estimating how much more toxin might be lost to the medium during filtration.

Efficiency of extraction: Assays of digested pulp following room temperature extraction with 1 M acetic acid, the standard conditions chosen for the survey, consistently indicated that less than 5% of the recoverable toxicity remained. This is clearly not a significant amount with respect to the present method, but was generally larger than would have been expected on the basis of free liberation of toxin and its dilution (generally five washes of about five volumes each, ideally 0.003% residual). Whether the discrepancy is due merely to inefficient extraction or to a refractory component of toxicity is not clear.

Alteration of composition: Early experience had shown extraction with hydrochloric acid to be treacherous. If the total amount of acid was small with respect to the buffer capacity of the system, composition was not altered but it was difficult to obtain efficient extraction. Extraction with sufficient 0.05 M aqueous HCl at room temperature or with hot 80% ethanol acidified with HCl (Alam *et al.*, 1979) reduced residual toxicity to a low value but led to serious alterations in composition. Room temperature extraction with either 80% ethanol acidified with acetic acid, or with 0.03 M aqueous HCl in which the extract was buffered with ammonium acetate prior to freeze-drying, caused no alteration; this implicated the HCl, particularly the excess HCl during freeze drying, as the component responsible. In light of this, it is interesting to recall an early paper by Hashimoto and Migata (1950),

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warning about the formation of artifacts by extraction with solvents that were acidified with HCl. Although, as they recognized, their observations were not related to paralytic shellfish poison, there is an element of prescience in their report.

II.B.8d. Interpretation of Chromatographic Results

As noted earlier (section II.B.3a), elution volumes of the toxins with the BioGel P2/acetic acid system were quite consistent for chromatography of *Protogonyaulax* extracts, but differed for the purified group A and B toxins. The shift in elution position, which appears due to a matrix effect, is rather fortuitous since the elution position of the purified group A and B toxins is about the same as that of the bulk of inert material in the extracts. If the matrix effect did not occur, there would be little resolution of these toxins from inert material and TLC would be impractical.

Sensitivity of the method is limited by the amount of resuspended, dried extract that can be loaded on the column, given the constraint that the effluent fractions are to be directly evaluated by the Buckley spot test and TLC. The sensitivity can be increased greatly if the fractions are concentrated prior to evaluation, although this is relatively cumbersome. Based on the chromatography of a standard mixture which contained 0.4 micromoles of each toxin except $\underline{\underline{10}}$ and $\underline{\underline{12}}$ and in which the spots in the resulting TLC were faint but still clearly visible, the method employed in this study can probably detect toxins at the level of 10 micromouseunits per cell with confidence, with an extreme detection limit of about 1 micromouseunit per cell.

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Interpreting the resulting chromatograms required appreciation of the chemistry of the toxins. The appearance of a carbamate toxin might imply either its presence in the dinoflagellate or hydrolysis of the corresponding sulfamate. The appraisal is, unfortunately, strictly a matter of seasoned judgement which could be equivocal in marginal cases. Fortunately, most cases were clear cut with a given toxin being either clearly present or absent.

Epimerization is also a problem. Although, at low concentrations, 6 gives a single spot on TLC, a second spot at the R_f of 4 was generally apparent at the concentrations encountered in LC of the extracts. However, subsequent column chromatography of the several fractions of 6 giving this second '4' spot gave only a single 6 peak, the more concentrated fractions of which again gave the second '4' spot. The second spot therefore appears to be merely trace epimerization of 6 to 4 during application and drying at the origin of the TLC plate, which becomes apparent at high concentrations of 6.

When conducted carefully, this analytical method gives a reliable estimate of sulfamate vs. carbamate toxins in the cells. Determining the relative amounts of the 11α- and 11ß-hydroxysulfate epimers remains problematic.

II.C. Variations in Toxicity and Toxin Composition

II.C.1. Introduction

The previous sections have established that *Protogonyaulax* could be cultured from several locations along the Alaskan coast and have

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described the array of saxitoxins. The following sections will first explore how toxicity and toxin composition vary in one clone (PI07) under different circumstances and then, having established the range of variability, will survey composition among several isolates.

In the following it is important to remember that, with the methods used, the observable properties (toxicity or toxin composition), are 1) the properties of a population, therefore not discriminating between a property uniform among all cells of the population and a property of differing population components; 2) the toxin recoverable in the cells and then extractable from them, therefore excluding leakage; 3) the substances that are toxic, or are rendered toxic, thus excluding structural or biosynthetic relatives that happen not to be active; and 4) reservoirs, rather than the rates of production and other processes of which the levels observed are the result.

All determinations of toxicities of cells, extracts, or fractions in the following experiments were preceeded by treatment to insure full Proctor enhancement (section II.B.2), apparently corresponding to the complete hydrolysis of the less potent sulfamate to the more potent carbamate toxins (II.B.5).

II.C.2. Variations in the Toxicity of Cells of Clone PI07

II.C.2a. Preliminary Carboy Experiments

To explore the effects of nutrient deprivation on cell growth and toxicity, three carboys were innoculated with about 500 cells/mL PI07 and grown at about 10°C. The nitrate enrichment had been omitted from

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the medium in the second carboy and phosphate from the third. By day 12 following innoculation, the increase in cell numbers in the carboy with normal medium had slowed considerably, while that in the carboys deprived of nitrate or phosphate had ceased days before. While the cells in all three cultures remained vigorous, those in the two deprived carboys developed morphologies which proved to be characteristic of the respective limitations. The surface of the nitrate limited cells became quite warty, obscuring the normal profile. The phosphate limited cells were distinctly larger than normal and seemed plump, without apparent changes in surface texture. Table 7 summarizes cell numbers and toxicity after 12 days and again after 27 days, at which point all three cultures still appeared reasonably healthy. Both the nitrate and phosphate results were unexpected. The increase in toxicity with phosphate deprivation was investigated further in vat cultures, described in the next section. The nitrate result was surprising since there had been conjecture that the toxins, due to their high nitrogen content, might serve as a nitrogen storage product. However, the toxin content of cells under acute nitrogen limitation at day 12 was no less than that of cells that were still growing.

II.C.2b. Vat Studies

Detailed studies of growth and toxin content were conducted in 500 liter vats which permitted frequent sampling without perturbing the system. The results are summarized in Figures 26-31. While the use of the vats had several advantages, they were sufficiently deep that light limitation became apparent at fairly low cell concentrations.

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Table 7. Changes in cell toxicity with nutrient depletion. Preliminary carboy experiments.

	Days after innoculation			
	12		27	
Medium	cells/mL	toxin/cell[1]	cells/mL	toxin/cell[1]
f'	7,800	290	16,300	150
f'-N[2]	3,100	270	3,200	160
f'-p[3]	3,200	890	3,000	770

[1] Toxin content in micromouseunits/cell.

[2] Medium f' with nitrate enrichment omitted.

[3] Medium f' with phosphate enrichment omitted.

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In the graphs of toxin content per cell, toxin content is expressed as micromouseunits per cell and displayed on a logarithmic scale to simplify interpretation. The major component of uncertainty in these values was due to the mouse bioassay, for which the 95% confidence limits were estimated to be ±23%. On a logarithmic scale, this corresponds to an interval that is independent of magnitude. A bar near the margin of each toxicity graph, corresponding to ±20%, is shown to simplify appraisal of apparent trends. In addition, though in a strictly qualitative sense at this point, the logarithmic scale is suited to representation of variations in toxicity because there would seem to be no meaning to 'zero toxicity per cell' in an intrinsically toxic organism, and because it seems that the factor by which toxicity varies is more significant than the absolute magnitude. However, unlike the use of the logarithmic scale in growth curves, no formal quantitative relationship is implied.

A series of vats (not shown) grown using a range of aeration intensities substantially above and below that normally used showed little difference in growth or toxin content, indicating that the range normally maintained was satisfactory and well below that found by White (1976) to attenuate growth in *Gonyaulax excavata*. Another series (Figures 26a, b, and c) in which the medium pH was maintained substantially above and below the normal range similarly showed little effect.

Growth at temperatures ranging from 6 to 12°C showed a reduction in growth rate at lower temperatures (Figure 27a) accompanied by a substantial increase in toxin content (Figure 27b). A series grown with various reductions in the initial medium phosphate (Figure 28a) showed

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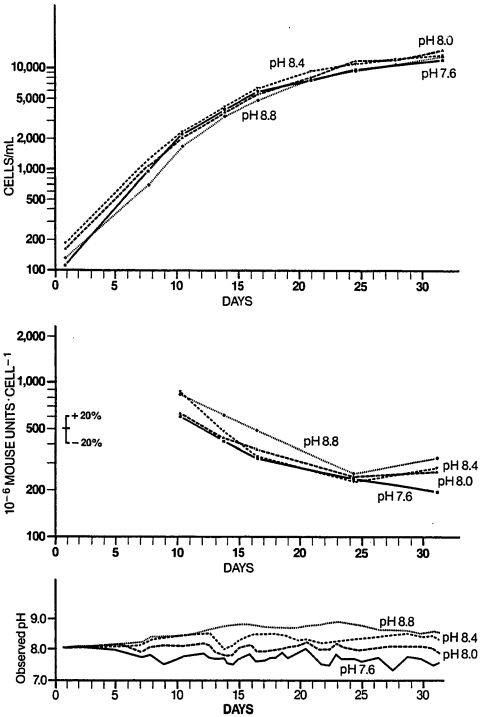

Figure 26a (top). Variation of growth with pH.

Figure 26b (middle). Variation of cell toxicity with pH.

Figure 26c (bottom). Observed medium pH.

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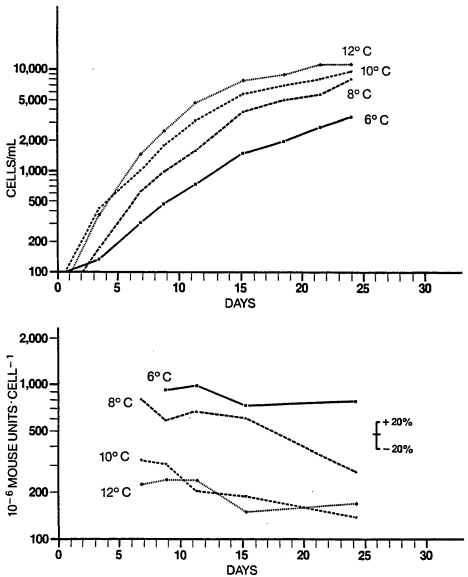

Figure 27a (upper). Variation in growth with temperature.
Figure 27b (lower). Variation in cell toxicity with temperature.

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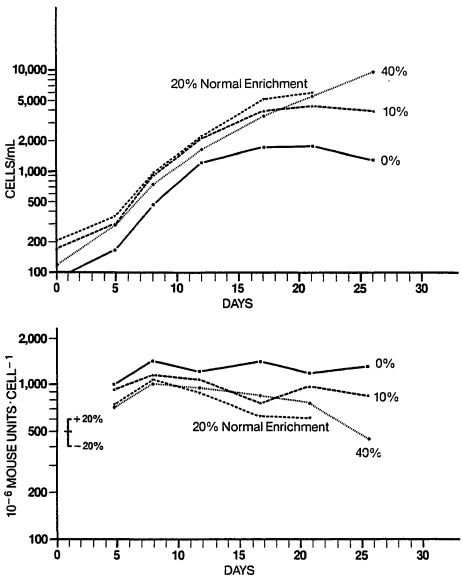

Figure 28a (upper). Variation in growth with initial medium phosphate.

Figure 28b (lower). Variation in cell toxicity with initial medium phosphate.

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that slightly higher toxicities (Figure 28b) resulted from the most acute phosphate limitation.

That growth attenuation in both cases corresponded to increased toxicity appeared to support the conclusion of Dupuy (1968) and Proctor *et al.* (1975) that toxin content was inversely related to growth rate. To evaluate this, a series of vats was grown in which it was intended to vary growth rate by varying light intensity. Unfortunately, the lowest light level chosen did not result in a dramatic attenuation of growth rate (Figure 29a), although growth in all four was eventually attenuated by shelf-shading. Toxin content (Figure 29b) does not show a simple trend at the outset, although in the later stages (more clearly in the re-plot, Figure 30b) growth attenuation by shelf-shading does correspond to decidedly lower toxicities. It therefore does not appear that growth rate or attenuation *per se* is the determining factor in toxin content. A similar conclusion was reached by White (1978), who observed variations in both the growth rate and toxin content of *Gonyaulax excavata* at different salinities, but saw no causal relationship between the two.

The data to this point do, however, suggest a possible inverse relationship between cell number and toxicity. A decrease in toxin content per cell as cultures mature has been noted in most reports (Prakash, 1967; Dupuy, 1968; Bates *et al.*, 1978), although only the more careful studies (White and Maranda, 1978; White, 1978) have discerned, as is apparent here, that the decrease begins well before the end of log phase growth. Comparing cultures grown under different conditions only on the basis of growth vs. time is also slightly misleading. A complementary,

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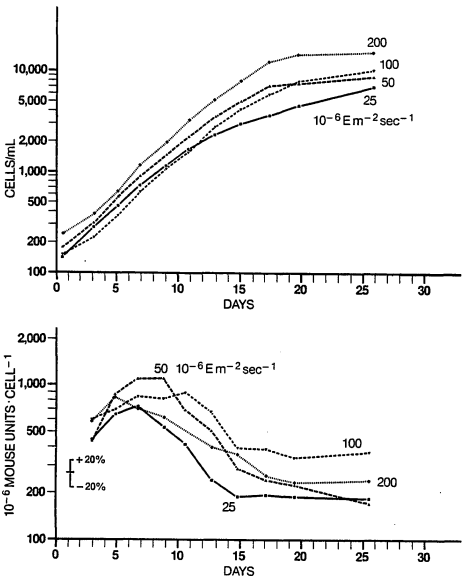

Figure 29a (upper). Variation in growth with light intensity.

Figure 29b (lower). Variation in cell toxicity with light intensity.

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though similarly imperfect, perspective is offered by using cell number itself as the progressive coordinate. The results from the previous series are re-plotted in this way in Figure 30 and support the impression that there is an inverse relationship between cell number and toxicity, although other trends are still apparent. Given the amount of nitrogen in the toxin structures it was necessary to consider the possibility that, despite the earlier finding that toxicity was not reduced by nitrate deprivation, the inverse relationship was due to nitrate depletion in the medium. A series of vat cultures grown to a phosphate limit (10% of the normal enrichment, Figure 31a) with the levels of nitrate enrichment varying by a factor of 32, showed little difference in toxicity (Figures 31b, c) with the nitrate concentrations used. While this is a rather primitive approach to nitrogen metabolism and further experiments with other nutrient concentrations might reveal a difference, this one experiment does not argue for nitrogen depletion as the cause of the inverse relationship.

Packed cell volume was also monitored during the culture experiments. Some of the variation in toxin content per cell appears to correspond to variations in cell volume, the general trend being that considering toxicity per cell volume rather than per cell reduces but does not eliminate the differences in potency. The data are of limited utility, however, because extraneous material, primarily shed thecae, interfered with precise determinations. The problem is particularly acute because thecal ecdysis is a common response to some of the same stresses that appear to increase toxin content, so that the data that would be the most

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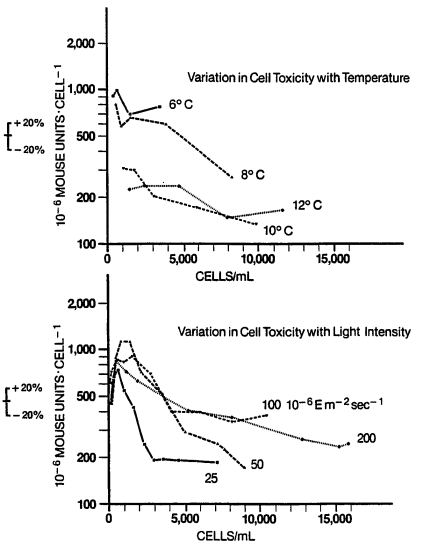

Figure 30a (upper). Variation in cell toxicity with temperature.

Figure 30b (lower). Variation in cell toxicity with light intensity.

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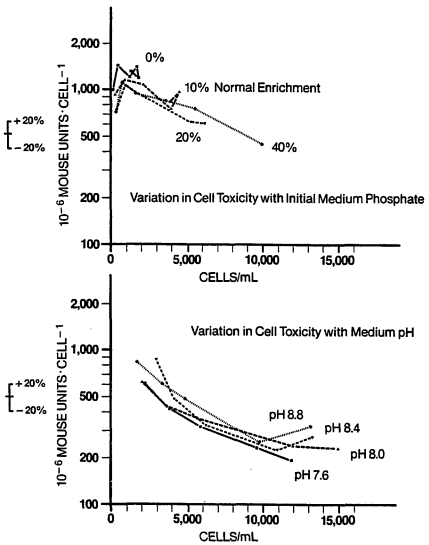

Figure 30c (upper). Variation in cell toxicity with initial medium phosphate.

Figure 30d (lower). Variation in cell toxicity with medium pH.

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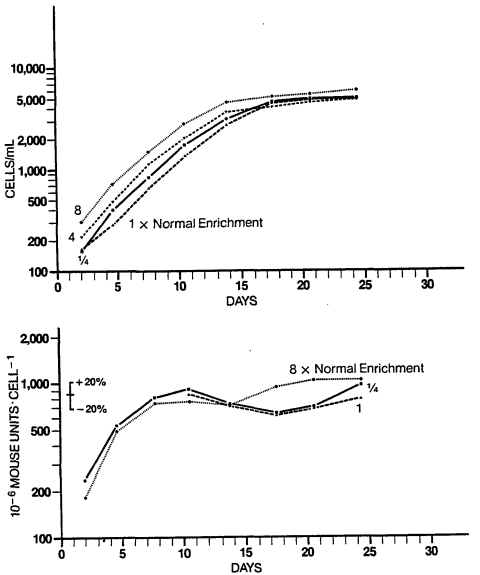

Figure 31a (upper). Variation in growth with initial medium nitrate.

Figure 31b (lower). Variation in cell toxicity with initial medium nitrate.

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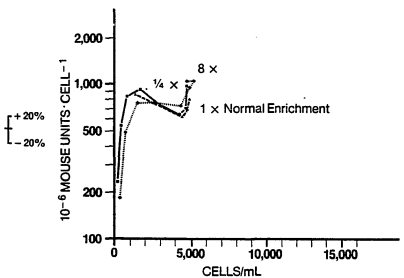

Figure 31 c. Variation in cell toxicity with initial medium nitrate.

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interesting tend to be the least reliable. Despite this limitation, scrutiny of the toxicity per cell volume data (not shown) indicates that 1) the differences between toxicities of cells grown in high and low phosphate media may be largely due to an increase in size with phosphate deprivation, but 2) the decrease in toxicity with growth of these and other cultures is apparent also on a cell volume basis, and 3) the differences in toxicity with temperature are not substantially altered by conversion from a cell number to a cell volume basis. White and Maranda (1978) observed that cells of *Gonyaulax excavata* in batch culture, although decreasing in size as the culture matured, decreased in toxicity on both a cell number and cell volume basis.

There is the distant possibility that a substantial amount of toxin leaks from the cells when they are sampled, that their permeability varies with growth conditions, and that the observed variations in toxicity are therefore not in the actual toxin content of the cells in culture but only in the amount that is lost during handling. This seems improbable, but cannot be formally excluded. If it is not the case, however, then the results show substantial variations in toxicity both during the growth of batch cultures and with variations in growth conditions.

These variations, and the high levels of toxicity attained, suggest that the amounts of toxin available from *Protogonyaulax* may be substantially higher than has been appreciated. Past estimates of *Protogonyaulax* toxicity have generally been based on cells grown under conditions that were convenient and resulted in good growth. While the enriched

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media and constant illumination employed here are hardly 'natural', the particular conditions that induced higher toxin content, low phosphate and low temperature, are closer to the 'natural' range of these parameters than those generally used.

The variations during growth suggest caution in assigning values for the intrinsic toxicities of strains within narrow limits, although the range of reported values substantially exceeds the variability observed here. Schmidt and Loeblich (1979) found that cultures of different strains of *Gonyaulax tamarensis* ranged from non-toxic to about 140 micromouseunits/cell. Some strains are thus clearly more toxic than others although, in view of the present findings, it would seem wise to re-evaluate the strains reported to be non-toxic under the conditions shown to induce high cell toxicity. Insofar as natural blooms resemble batch growth, it is likely that cell toxicity will vary as a bloom develops such that quantitative correlations between *Protogonyaulax* abundance and shellfish toxicity should not be expected.

II.C.3. Variations in Toxin Composition in Clone PI07.

Given that toxicity in PI07 is due to several toxins and that gross toxicity varies substantially with growth conditions, the next question is whether changes in toxin composition relate to the changes in overall toxicity or whether the relative composition is fairly constant. Analyses of PI07 grown at high and low temperature, to phosphate limitation, and sampled throughout the growth of a batch are summarized in Table 8. Aside from a slight reduction in the relative amounts of $\underline{7}$ and

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Table 8. Toxin composition of clone PI07 under various growth conditions.

Culture	Extraction[1]			Chromatography[1]						Composition						
	Seawater supernatant	Pulp	Extract	Applied suspension	Early	A	B	C	Late	1	2	3/5	4/6	7	8	9/11
#1: young (1990/mL)																
	25	12	460	420	12	46	210	150	6	+			+	+	+	+
#2: mid-cycle (4320/mL)																
	9	4	370	340	<6	32	120	110	8	+			+	+	+	+
Old (8850/mL)																
	9	.3								+			+	+	+	+
	8	.4								+			+	+	+	+
#3: phosphate limited (10% of normal enrichment)																
										+			+	+	+	+
#4: warm (15-20°C)																
	1	4								+[2]			+	+	+	+[2]
#5: warm (15-20°C)																
	28	5	[3] 48[4]	87	<2	1	34	29	<1	+[2] +[2]			+ +	+ +	+ +	+[2] +[2]

[1] Values are toxicities in micromouseunits/cell which, due to the imprecision of the mouse bioassay, have an uncertainty on the order of ±20%.

[2] Weak.

[3] Concurrent cell assay: 150 micromouseunits/cell.

[4] Concurrent cell assay: 98 micromouseunits/cell.

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9/11, composition appears to be rather consistent. It is possible that a more quantitative study will reveal significant patterns but, at this point, the data do little to suggest biosynthetic relationships among the toxins. If anything, the uniformity of relative composition argues for variations in gross toxicity being due to leakage. While the data fail to illuminate biosynthetic pathways, they do imply that composition, in contrast to gross content, is a fairly stable characteristic of an isolate.

II.C.4. Patterns of Toxin Composition Among Isolates

Analyses of several isolates for toxin composition are summarized in Table 9. Substantial differences were apparent in strains from different regions, while each strain gave a uniform pattern in successive runs that was identical to that of isolates from neighboring areas. Fortunately, the differences were rather clear. Toxins were either present or absent, with few cases that were marginal. As noted before, trace hydrolysis of the sulfamate toxins can lead to faint spots for the carbamate toxins. While this could have been a problem in discriminating between the Haines group, in which 3/5 accompany 4/6, and the Icy Straits group, which lack 3/5, the amounts observed permitted assignment with confidence.

As pointed out before, the levels of 10 and 12 were low at best, faint but detectable in PI07 run at high concentrations. They were not observed in other strains but, at the concentrations run, would not have been expected to be detectable.

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Table 9. Variation in toxin composition among strains.

Isolate	Culture Extract	Extraction[1] Seawater supernatant	Extraction[1] Pulp	Extraction[1] Extract	Chromatography[1] Applied suspension	Chromatography[1] Early	Chromatography[1] A	Chromatography[1] B	Chromatography[1] C	Chromatography[1] Late	Composition 1	2	3/5	4/6	7	8	9/11
DH07	1 1	8	.3										[3]	+	+		+
	2	3		32	58	<7	10	3	28	<4			[3]	+	+		+
LH01	1 1	12	.2	65	76	<7	12	4	51	<1			[3]	+	+		+
	2												[3]	+	+		+
PW06	1										+				+[2]		
	2	<2	2	50	53	<7	51	<2	<4	<5	+				+[2]		
EC06	1	73	.8	160	130	<7	23	68	53	<6		+		+	+	+	+
	2											+		+	+	+	+
BC07												+		+	+	+	+
PI07	1	9	4	370	340	<6	32	120	110	8		+		+	+	+	+
	2	25	13	460	420	<12	46	210	150	6		+		+	+	+	+
HG01	1 1			110	110	<8	21	52	16	<4		+	+	+	+	+	+
	2											+	+	+	+	+	+
	3											+	+	+	+	+	+
HG27	1 1											+	+	+	+	+	+
	2											+	+	+	+	+	+[2]
KN03	1	3	.5	34	37	<5	2	18	18	<4		+		+	+	+	[3]
	2											+		+	+	+	+
SF01	1 1	11		54	61	6	50	.8	25	<2	+			+	+		
	2										+			+	+		
	3										+			+	+		
SF05											+			+	+		

[1] Values are toxicities in micromouseunits/cell which, due to the imprecision of the mouse bioassay, have an uncertainty on the order of ±20%.

[2] Relatively weak.

[3] Too weak for reliable detection.

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The observed pattern of toxin composition has several implications:

1) It appears that the observed distributions are a real and reproducible property of an isolate, not an artifact of culture or analytical methods.

2) The sulfamate toxins, particularly $\underline{4}/\underline{6}$, are a dominant feature of the toxins contained in *Protogonyaulax* from the entire range sampled.

3) Although the number of samples is relatively small, the pattern strongly suggests regional uniformity in toxin composition, implying geographically defined populations of *Protogonyaulax* that are uniform in the composition of the toxins they contain.

4) Among the 11-hydroxysulfate compounds, the β epimers consistently predominate, in contrast to the predominance of the α epimers *in vitro*. This suggests that the nature or circumstances of the toxins differ *in vivo*.

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III. A SUMMARY OF CAVEATS

The results of this work have significant implications. It is important, however, that they be viewed with due caution:

1) The study, particularly the work on toxicity vs. growth conditions, is preliminary, designed for the most part to provide background for definitive work.

2) The organisms used in this study were of necessity select volunteers, isolated from among those that grew well in the laboratory under the conditions chosen. This could well have selected populations that are not representative of those selected by natural conditions.

3) Cultures were in most cases grown at nutrient and cell concentrations well above those found in nature. The increase in toxicity as phosphate concentrations were reduced points out that artifacts can result from such factors. The inverse relationship between cell concentration and toxicity could imply that, at the substantially lower cell concentrations found in nature, toxicity could be still higher.

4) Variations in toxicity with growth conditions were studied in detail for only one clone. However, extrapolation to *Protogonyaulax* in general seems likely to be appropriate, on the basis of fragmentary data from other strains in this and other studies.

5) The variation in toxicity during batch growth indicates that factors not yet defined are significant in determining the level of cell toxicity.

6) The methods used to evaluate toxin content per cell would not be able to discriminate between a uniform change in the population and a shift in

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the relative abundance of more and less toxic population components. If, for instance, cell toxicity were a great deal higher just after division, conditions that prolonged this phase would increase the observed toxin content per cell in the culture.

7) Although a large and reproducible amount of toxin is recoverable in *Protogonyaulax* cells, all the data suffer from uncertainties about how much toxin leaks out, either under normal circumstances during the life of cells, or as a result of unavoidable abuse during sampling. Leakage has frequently been mentioned as a possible basis for observed variations in cell toxicity. If this occurs in culture and in nature, then the toxin remaining in the cell is indeed what is available to filter feeders and the trends may be properly reflected by data such as section II.C.1. However, it is conceivable that the differences seen reflect primarily a variation in the cell's resistance to loss during centrifugation and that cells not thus abused differ little in toxicity.

8) The bacterial population in the cultures was neither controlled nor monitored. It is possible that the observed variations in cell toxicity were due to effects on antagonists or synergists, rather than on the organism itself. The patterns in toxin composition could also be due to entities other than the *Protogonyaulax* themselves but, given the observed geographic pattern, it is most likely that the compositions correspond to something found in nature and, if the source is not solely the dinoflagellate, then it is an isolable assemblage characteristic of the location sampled.

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9) The hypothesis regarding geographic patterns of composition is based on a relatively small number of samples. However, it is difficult to support alternative hypotheses with the data.

10) Toxin analyses were of the thecate, vegetative stage while it appears that the hypnozygote, which might differ in toxin composition, may also be a significant source of toxin to the shellfish.

11) It is possible that various life cycle stages exist within nominally vegetative cultures, and that some differences in composition were due to similar organisms in different stages. This seems unlikely, however, given the pattern of regional uniformity.

12) The analyses were of dinoflagellates. Comments with respect to shellfish are extrapolations. However, the recent report of toxins C1 (4) and C2 (6) in Japanese oysters (Onoue *et al.*, 1981) indicates that the 21-sulfo toxins occur and are relevant in nature.

13) The toxin analyses were necessarily only of the material that could be recovered in the cells and then extracted under conditions that appeared suitable for the compounds present. The amount of toxin not removed from the cells by acetic acid extraction, subsequently estimated by digestion with HCl, appears relatively small and does not likely alter the composition results. The amount lost from the cells to the medium, however, could not readily be estimated and may greatly exceed 10% of the amount recovered. If the composition of the toxin thus lost differs significantly from that of the remainder, it would introduce serious bias into the results. However, the uniformity seen in replicate determinations argues against such bias.

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14) There were few internal standard runs, so there is little control over possible systematic losses of particular toxins. However, the generally good recoveries during preparative manipulations argue against serious losses as, again, does the consistency of replicates.

15) It is clearly possible not only that structural modifications of saxitoxin beyond the three described here could exist in *Protogonyaulax*, but that their chemical nature might systematically preclude detection by these methods, being hydrolyzed or otherwise altered during extraction and manipulation or present but not detected by the Buckley spot test. Given the ratios of 11α- and 11ß-hydroxysulfates *in vitro* and *in vivo*, it would certainly appear that the circumstances of the toxins *in vivo* differ somehow, and that the 11α compounds may not be present. Furthermore, since the chromatographic method does not approach baseline resolution of the twelve known toxins, the profile and recovery can hardly exclude the presence of a lurking, undetected component.

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IV. DISCUSSION

IV.A. Toxins and the Dinoflagellate

IV.A.1. Taxonomy and the True Source

While the toxins can obviously be obtained from *Protogonyaulax* cultures, *Protogonyaulax* are not their sole source if indeed the source at all. Alam *et al.* (1978) have obtained saxitoxin (1) and three related substances from the blue green alga *Aphanazomenon flos-aquae*, demonstrating that the pathways for the synthesis of the toxins occur in procaryots. Silva (1962) has suggested that toxicity in *Gonyaulax tamarensis* is due to endosymbiont bacteria. The occurrence of the toxins in Xanthid crabs (Koyama *et al.*, 1981; Yasumoto *et al.*, 1981), where it is difficult to rationalize a source, may be evidence for toxigenesis other than by *Protogonyaulax*.

In an early study of the toxicity of *Gonyaulax catenella*, Burke *et al.* (1960) claimed their cultures to be axenic, as did White (1978) in more recent work on variations in the toxicity of *Gonyaulax excavata*. In the present study, no precautions were taken to exclude bacteria from the cultures, but the regional pattern of toxin composition shows that whatever made the toxin in the cultures originated in the samples and incubation plates from which the strains were isolated. Both toxic and non-toxic dinoflagellates were on several occasions isolated from the same incubation plate. If something other than *Protogonyaulax* itself is the source, then it must have originated with the samples and associated only with dinoflagellates assignable by morphology to *Protogonyaulax*.

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While a symbiont could well exhibit such selectivity, these observations limit the possibilities.

In a broad survey of dinoflagellates for PSP toxins, Schmidt and Loeblich (1979b) found toxicity only in those isolates that would be assigned to the genus *Protogonyaulax*, with the exception that trace levels were detected in *Gonyaulax monilata* and may have been detected in *Gonyaulax polyedra*. On the other hand, they found little or no toxicity in *Gonyaulax tamarensis* from Plymouth, England, in agreement with earlier results (Prakash, 1967). Yentsh *et al.* (1978) report blooms of *Gonyaulax tamarensis* in Maine that appear devoid of toxicity. Considering, however, the conditions under which the 'non toxic' organisms grew and the results presented in section II.C.2, it is not clear whether these findings reflect an intrinsic lack of toxicity or simply its suppression below detectable levels.

The geographic pattern of toxin composition found in the present work (section II.C.4) indicates that there are at least five regional, distinct populations of the toxin source organism along the northeast Pacific rim. Unless toxigenesis can be attributed to associated organisms, such as symbiont bacteria, the currently accepted species do not account for the variation among dinoflagellate populations assignable to the genus *Protogonyaulax*. Coupled with the difficulty of making meaningful species assignments on the basis of morphology (section II.A.3), the results suggest that a taxonomic reassessment of the genus is in order.

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IV.A.2. Metabolic Origin and Function of the Toxins

IV.A.2a. General Considerations

Concern over the toxins leads to a rather narrow view of secondary metabolism in dinoflagellates. Surely there are lots of other interesting compounds. What we see in looking at toxicity is only a component of the whole. But most are relatively difficult to focus on, in contrast to the toxins which, because they can be detected with a relatively simple bioassay, can be detected at fairly low levels and followed through purifications. Toxicity, in a sense, provides a window on general nitrogen metabolism in the source organism. It is important to recognize the limitations on the view provided. First, these are likely only a small component of the whole inventory of nitrogenous secondary metabolites. Second, not all substances with structural or metabolic relationships to saxitoxin are toxic. The 21-sulfo toxins point out how misleading toxicity can be as a sole criterion. Even more serious, although they have not yet been reported in natural material, are the compounds in which the 12-ketone has been reduced (Boyer, 1980; Koehn *et al.*, 1981) or in which C-12 bears no substituent (Kishi, 1980). Both have little or no toxicity and are unlikely to be rendered toxic by simple treatments. Toxicity is obviously of little use in detecting the nontoxic compounds that preceed or follow the toxins in the metabolic sequence.

It is important to realize that what is observable is not synthesis but pools and that they, rather than suggesting that *Protogonyaulax* synthesizes the toxins while other dinoflagellates don't, formally indicate only that in *Protogonyaulax* toxins accumulate to detectable levels.

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Cultures obviously produce the toxins. Cells contain them. The content can be measured and, on a cellular basis, is the result of production and other processes that increase or decrease the apparent toxicity of cells. The problem is dissecting out the production component and establishing what is responsible for it.

The measurable intracellular toxicity or toxin composition are the results of:

1) relative rates of synthesis of the toxins and other cellular material;
2) interconversions among the toxins which, due to their differing molar activities, will alter gross toxicity;
3) the rates of metabolism or degradation of the toxins to non-toxic substances, and leakage of the toxins from the cell.

Leakage clearly occurs to some extent, but it is difficult to determine the kind and amount of toxin lost. If leakage is negligible under normal circumstances, then the observed patterns of toxin content and composition must be explained in terms of metabolic processes within the cell and, conversely, offer some basis for inferences regarding those processes. Many of the observations could be explained by a substantial leakage component. If this occurs, however, inference regarding biosynthesis is difficult.

IV.A.2b. The *in vivo* State of the Toxins

While the results of the present study strongly suggest that the array of twelve toxins represents the complete set extractable from

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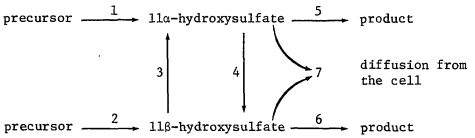
Protogonyaulax, two observations suggest that, *in vivo*, the state of the toxins may somehow differ.

First, in contrast to the predominance of the 11α-hydroxysulfates in equilibrated mixtures, the analyses consistently showed the 11β-hydroxysulfate epimers to predominate in fresh extracts (sections II.B.8 and II.C.4; Fix Wichmann *et al.*, 1981a). The paths relevant to this are:



The simplest hypotheses are that the β epimer is the form synthesized (2 occurs, 1 does not) and/or that the β pool is stabilized (4 is greater than 3). However, the data show only that the relative size of the β pool is larger than would be expected from *in vivo* behavior, requiring only that the rates out of the α pool (either conversion 5 or diffusion 7) be large enough, with respect to the rates in (synthesis 1 or epimerization 3), to suppress the observed level relative to the β pool.

Second, experience *in vitro* shows the toxins to be susceptible to oxidation except when the pH is kept well below 7. Oxidation of the toxins to low levels would therefore be expected *in vivo*, unless the toxins differ in chemical nature, are kept in a microenvironment either free of oxygen or at low pH, or are synthesized at a rate that is large with respect to the rate of oxidation.

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While both observations might indicate a difference in the nature of the toxins *in vivo*, the alternative explanations are as plausible.

IV.A.2c. Inference From Variations in Toxin Content and Composition

The physiological studies, evaluating the variations in toxin content with growth conditions, were undertaken in part with hopes that the variations would suggest how the toxins fit into the cell's metabolism, possibly illuminating biosynthetic relationships. While these studies succeeded in demonstrating that cell toxicity was not merely a consequence of growth rate, they failed to define what the causal factors were. Different conditions clearly made cells more or less toxic, but variations also occurred without recognizable cause. That low temperature and phosphate concentration were able to induce higher toxicities was encouraging.

If these factors were altering toxicity by a direct effect on the biosynthetic pathways, then differences in composition might be observable at high and low toxicities. Unfortunately, the differences in composition were relatively small. Better quantification might reveal useful patterns but, at this point, the data are not inconsistent with a simple change in leakage rates, affecting the various components uniformly. In observing similar patterns of variation in the toxicity of *Gonyaulax excavata* with differences in salinity, White (1978) considered variations in toxin leakage to be a significant possibility.

If leakage is a major factor in determining both the levels of toxicity and the observed ratio of 11α- and 11ß-hydroxysulfate epimers,

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then lower cell toxicity (i.e., higher leak rates) should correspond to a higher ratio of 11β- to 11α-hydroxysulfates, if it is assumed that the 11β epimer is the form initially synthesized. This was indeed the case for the group C toxins of clone PI07 in two cases. In the first, a culture grown at high temperature yielded cells with a relatively low (90 micromouseunits/cell) toxicity, and a molar ratio of $\underline{\underline{6}}/\underline{\underline{4}}$ of about 7. Cells grown at lower temperature to a moderate toxicity (420 micromouseunits/cell) had a $\underline{\underline{6}}/\underline{\underline{4}}$ ratio of about 3. Given the uncertainties in the method these ratios must be viewed with great caution, but they are at least not inconsistent with the hypothesis. In other respects, the general uniformity of toxin composition, at a very crude level of appraisal, in PI07 grown to high or low toxicity is also consistent with leakage as a major determinant of toxicity.

As a hypothesis regarding toxin content and composition, leakage is utterly lacking in aesthetic appeal and wreaks havoc with efforts to infer the nature of active processes in toxin metabolism. However, it must be considered as an alternative. Unfortunately, data at this point cannot exclude it and, if anything, tend to support it.

In the majority of cases, the toxicity of *Protogonyaulax* cells varies substantially during the growth of batch cultures, for reasons that are not yet apparent. Studying *Chlamydomonas* sp. in batch culture, Ryther (1954) found that, while respiration decreased twofold during the growth of a culture, photosynthesis decreased 20 fold. Beardall *et al.* (1976) found that *Gonyaulax tamarensis* (Martin isolate, found in other studies to be toxic) was like other algae tested, in that

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photosynthesis was initially high, decreasing during batch growth. This pattern coincides with that for cell toxicity found in the present study (section II.C.2) for clone PI07, and elsewhere (White and Maranda, 1978; White, 1978) for related strains. Given that cell toxicity was reduced by lower light and higher temperatures (section II.C.2), it is tempting to speculate that relatively high rates of photosynthesis and/or low rates of respiration contribute to high toxin content.

The metabolic origin and function of the toxins remains obscure. The different toxin compositions observed in section II.C.4 surely contain information about biosynthetic relationships that will become clear in retrospect. At present, they only suggest that the levels observed are not essential, since most toxins are variously present or absent.

The structures of the toxins strongly suggest a link to purine metabolism, the skeleton being formally a reduced purine. However, there is little precedent in known purine biosynthetic pathways for the reduction of purines (Henderson and Paterson, 1973; J. Frank Henderson, personal communication). Michaelson and Yentsch (1978) propose a relationship between the toxins and nucleotide polymers that is particularly attractive because it not only points to biosynthetic origins, but further explains the decrease in cell toxicity with growth in batch culture. Unfortunately, aside from the small amounts of toxicity found in the pulp assays (section II.B.8), data from the present study reveal no such component of high molecular weight toxin.

It has been suggested that the toxins might be a nitrogen storage product yet, in one experiment, cell toxicity was not reduced in a

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culture grown to a nitrogen limit. This suggests that the toxins are not accessible as a nitrogen reserve, and leads to the proposition (first suggested in a conversation with Blanch Meeson, University of California at Santa Barbara, and thus the 'Meeson hypothesis') that *Protogonyaulax* accumulate the toxins because they are deficient in the enzymes that would normally convert them to other substances. Carrying this a step further, present data cannot exclude the possibility that pathways for the synthesis of the toxins, rather than being a unique property of a few organisms, are rather widely distributed. In this respect it should be remembered that, as discussed by Geissman and Crout (1969), both squalene and shikimic acid are now recognized to be broadly distributed and the bases for major pathways, but were first thought only to be rare compounds, squalene being found in shark oil and shikimic acid in an Asiatic plant.

IV.B. Toxins and Substrates

IV.B.1. Introduction

IV.B.1a. The Perspective and the Reason

Selective binding is the key phenomenon to be understood in dealing with the saxitoxins. They are toxins only because they bind, selectively and rather efficiently, to a specific site on excitable membranes. They are a hazard largely because they accumulate in shellfish, due in part to relatively efficient, selective binding. A useful chemical analysis, which has yet proven an elusive goal, will likely depend on the development of *in vitro* systems that successfully mimic the

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selectivity attained in natural systems. While the use of saxitoxin (1) in combination with other substances as a local anaesthetic has already been patented (Adams and Takman, 1977), it would appear that more will eventually be achieved by elucidating the structural bases for toxin activity and developing analogs that employ them.

Despite the merits of this perspective, it serves at present as a framework more for questions than answers. While it is obvious that selective binding occurs and that it would be useful to understand the mechanisms, present information places only very general constraints on what those mechanisms might be. The tools available for investigating the mechanisms include competitive interactions and structural variations in either the toxin or binding site. To understand the toxin/binding site interaction, one ideally would define the structures of both. Explicit definition of the binding site structure is a distant goal, although some data have been obtained. At the first approximation, the toxin structures are well defined but the task now is to define their structures under relevant circumstances. This is a problem because, while we now have unambiguous structures for 1, 6, and 12 in the solid state, we have at this point only rudimentary information on the structures in solution and, when these are defined, will still face uncertainties about the effect of micro-environments at two levels. First, while the relevant properties of the toxins are known to be pH dependent, it is difficult to define *in situ* pH with certainty and, second, it is entirely possible that the conformation of the toxin on the binding site and relevant to the binding process differs from that which predominates in solution.

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IV.B.1b. Structure of the Saxitoxins in Solution

IV.B.1b.(1) Structural Variations

The structural variations of saxitoxin (1) for which data are available include 1) N-1-hydroxyl, 11-hydroxysulfate, and 21-sulfo derivatives, forming the array of twelve natural saxitoxins; 2) reduction of the 12-ketone hydrate to form α- and β-saxitoxinol (13, 14), dehydration of saxitoxin to the keto form (25), or complete absence of oxygen at C-12 (19); and 3) removal of the carbamoyl group to form the alcohol dcSTX (15), then esterification to dcSTX hemisuccinate (18).

IV.B.1b.(2) Dissociations and Net Charge

Extensive titration and NMR studies (Shimizu *et al.*, 1978b; Rogers and Rapoport, 1980; Shimizu *et al.*, 1981) have led to the conclusion that in 1 and 7 the C-8 guanidinium deprotonates with a pKa about 8.5, and the C-2 guanidinium with about pKa = 11.5. These values differ little in dcSTX (15), α- or βSTOH (13, 14), or αdcSTOH (17). The N-1-OH of 7 (Shimizu *et al.*, 1978b) deprotonates with pKa = 6.75. Dissociation constants and net charge of the ten remaining natural saxitoxins have not yet been determined. It is tempting to simply add substituent charges but (Larry Overman, personal communication) this could be misleading due to substituent effects on dissociation constants. Both dissociations and charge could in principle be inferred from chromatographic (section II.B.3) and electrophoretic (Fallon and Shimizu, 1977; Shimizu, 1979) properties, but the data at present are relatively sparse and the inferences neither precise nor consistent.

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The elution of 8, 9, and 11 from carboxylate resins with pH 7.5 buffer (section II.B.3b) suggests that the N-1-hydroxyl does indeed deprotonate well below pH 7.5, rendering them uncharged. On the other hand, electrophoretic evidence (Shimizu, 1979) suggests that 11 still has a slight net positive charge at pH 8.7.

IV.B.1b.(3) Ketone/Ketone Hydrate Equilibrium

The C-12 geminal diol generally observed in the saxitoxins in the crystalline state (sections II.B.6f and h; Schantz *et al.*, 1975; Bordner *et al.*, 1975; Fix Wichmann *et al.*, 1981b) and in solution is formally equivalent to a hydrated ketone. In ^{13}C-NMR (Rogers and Rapoport, 1980) and ^{1}H-NMR (Shimizu *et al.*, 1981) of 1 at high pH, new sets of peaks occur which have been assigned to the keto form (25). Shimizu *et al.* (1981) noted that the peaks became more intense as the pH was increased through the first pKa, but that the pH dependent shift of H-5 that occurred in the ketone hydrate through this range was not seen in the H-5 peak of the keto form. From this, they conclude that dehydration to the keto form occurs primarily after the C-8 guanidino is deprotonated. This is consistent with the earlier proposal (Schantz *et al.*, 1975) that under normal circumstances the equilibrium favors the ketone hydrate because it is stabilized by the adjacent guanidinium groups. Shimizu *et al.* (1981) do not discuss a second possibility, that dehydration of the ketone stabilizes the C-8 guanidinium, shifting its pKa above the range evaluated, but this seems unlikely in view of the relatively modest change in the first dissociation constant (from 8.22 to ca 8.55, Rogers

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and Rapoport, 1980) resulting from reduction of the 12-ketone to form 13 and 14.

In earlier chemical studies, Wong *et al.* (1971) had found that drying under extreme conditions (0.01 microns, 110°C) removed, reversably, one equivalent of water from 1. Experiments with ^{18}O-labelled water showed that a single oxygen was being removed and replaced. In subsequent x-ray studies on the crystalline ethyl hemiketal of 1 (20) (Bordner *et al.*, 1975), it was found that the ethanol oxygen was in the 12β position. Hydrogenations of the C-12 ketone under differing conditions all favor formation of the 12α alcohol (αSTOH) to varying degrees (Koehn *et al.*, 1981; Rogers and Rapoport, 1980; Shimizu *et al.*, 1981). Taken together, these observations suggest that the β side of C-12 is more accessible and that the equilibrium involves removal and replacement of the oxygen at the 12β position (oxygen 17).

Kinetic data on the equilibrium are lacking, although it was noted (Bordner *et al.*, 1975) that the half life of saxitoxin ethyl hemiketal (20) was 40 minutes at 20°C in D_2O.

IV.B.1b.(4) Conformation

Data currently available bear upon the tricyclic skeleton of the saxitoxins and rotation of the carbamate side chain around the C-6/C-13 bond. The orientation of the carbamate side chain beyond this, or that of the 11-hydroxysulfate, can at present only be inferred from models.

From NMR studies under conditions such that the species observed was the ketone hydrate, Niccolai *et al.* (1980) show the tricyclic

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framework of saxitoxin (1) to be rigid in solution and that its conformation corresponds to that found in the crystal structure (Bordner *et al.*, 1975).

Orientation around the C-6/C-13 bond can be inferred from NMR data for H-6 and the two H-13. The H-13 signals are distinct in all twelve natural saxitoxins (Table 4). The distinction is conserved in βSTOH (14) but in αSTOH (13) the signals merge (Koehn *et al.*, 1981; Shimizu *et al.*, 1981). In dcSTX (15) the signals merge but the distinction is restored in dcSTX hemisuccinate (18) (Ghazarossian, 1977; Koehn *et al.*, 1981). From slightly different lines of reasoning, both Koehn *et al.* (1981) and Shimizu *et al.* (1981) conclude that the 12α hydroxyl has little effect, while there is a substantial interaction between the side chain and the 12β hydroxyl, altering rotation.

To quantify the effect on rotation, Shimizu *et al.* (1981) calculated the C-6/C-13 rotamer populations from H-6/H-13 coupling constants. Those for STX (1) show the predominant rotamer to be that with O-14 anti to C-5. This corresponds to the orientation found in the crystal structure of 1 (Schantz *et al.*, 1975a; Bordner *et al.*, 1975), and to that found by Darling (section II.B.6f) in the crystal structure of 6. The coupling constants for βSTOH (14) were similar, indicating a similar rotamer population while those for both the keto form of STX (25) and αSTOH (13) differed, showing the predominant rotamer in both to have O-14 oriented down over the face of the six-membered ring, between N-1 and C-5 and further emphasizing the importance of the 12β hydroxyl. In the present work, H-13 chemical shift differentials and H-6/H-13 coupling constants

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differed little between each carbamate toxin and its sulfamate counterpart, suggesting that the presence of the 21-sulfo group has little effect on side chain rotation.

IV.B.2. Binding to Excitable Tissues

IV.B.2a. Kinds of Experiments and Data

Available data come from three kinds of experiments: 1) assays, such as the mouse bioassay, for *in vivo* potency; 2) electrophysiological, measuring the blockage of ionic fluxes in isolated tissue; and 3) binding, generally with radio-labelled toxins, measuring the amount of uptake or loss by isolated tissue. Each has its limitations.

It is difficult to extract information relevant to the binding site from any data that are not steady state, particularly from a dose response/death time assay such as the mouse assay, since the actual activity at the site may be obscured by differing rates of diffusion, non-specific binding, or other events along the way. Even in such preparations as the isolated squid giant axon, the active site is separated from the bathing solution by layers of cells that greatly attenuate diffusion. The significance of *in vivo* non-specific binding in the mouse bioassay is clear from studies by Wiberg and Stephenson (1960), who found that the pH of the solution injected ip greatly influenced assay results.

In binding experiments, the fundamental problem is to establish that the observed binding has anything to do with binding to the active site. This is generally approached by dissecting the observed curve of toxin concentrations vs. toxin bound into high affinity 'specific' and low affinity 'non-specific' components.

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Electrophysiological experiments relieve this ambiguity, clearly demonstrating a relationship between toxin concentration and blockage of the active site. In comparing the activities of analogs, however, it is difficult to differentiate between alteration of binding efficiency and alteration of the blocking efficiency of a bound species.

IV.B.2b. The Sodium Channel and Alterations of the Binding Site

The transmission of impulses along nerve and muscle membranes depends in part on a transient increase in permeability to sodium ions, which flow from a high concentration outside the membrane to a lower concentration inside the membrane. This transient conductance is presently modelled as the brief opening of a sodium channel, and the activity of the toxins is attributed to a specific, reversible blockage of the channel.

Research on excitable membranes leading to the concept of the sodium channel was, however, conducted primarily with tetrodotoxin (TTX), a substance obtained from puffer fish and several other animals that causes symptoms similar to PSP. Electrophysiological studies using TTX appearing during the period 1960 to 1965 (Narahashi, 1972) led to: 1) the initial concept of interference with sodium conductance, 2) the demonstration that TTX similarly blocked the flow of other monovalent cations (e.g., lithium) used in place of sodium in the bathing solution; 3) the observation that TTX was active on the external surface of the membrane but not on the inside; and 4) the observation that the binding was reversible. The initial *in vitro* work on STX (1) indicated that it

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was TTX-like (Kao and Nishiyama, 1965), acting along the nerve or muscle fiber (Evans, 1964; Kao and Nishiyama, 1965) rather that at the neuromuscular junction as had previously been stated. It is interesting, in view of the importance subsequently attached to the mechanism of binding, that there appears to have been a lag of several years between the suggestions that STX (1) and TTX both acted by the same mechanism and the first explicit proof, which appears to have been by Hille (1968a). While elegant kinetic studies by Wagner and Ulbricht (1975) and binding studies by Henderson *et al.* (1973) have indicated that the two toxins bind to the same site, more recent work by Kao (1981) using chiriquitoxin, a natural derivative of TTX, indicates that the two, though similar, are not identical in their action.

The toxin binding site appears distinct from the rest of the sodium channel mechanism. The work of Twarog and her associates (Twarog *et al.*, 1972; Twarog and Yamaguchi, 1975) was an early indication of this, showing that nerve preparations from several species of bivalves differed in their sensitivity to STX (1). Since Evans (1964) had shown that frog sciatic nerve perineural sheath was highly impermeable to the toxins, they took care to demonstrate that the differences in sensitivity were seen in desheathed nerve preparations and were therefore due to properties of the nerves themselves. It had been suggested that immunity to the sodium channel blockers would be found in nerves whose function was based on the flux of some other cation (e.g., calcium), but activity of the bivalve nerve preparations was shown to be sodium dependent. The implied existence of sodium channels immune to blockage by the toxins

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suggests that the functional parts of the sodium channel, responsible for both valving ion flux and selecting sodium ions, are distinct from the site responsible for binding the toxins. This was explicitly demonstrated by Spalding (1980), using frog muscle fibers that are normally susceptible to a saxitoxin (1) block. Treatment of the fibers with trimethyloxonium ion, which derivatizes carboxylate groups, left about 1/3 of the sodium channels active. Of these, 1/2 were no longer susceptible to blockage by saxitoxin (1).

There has been some progress in the isolation and purification of the saxitoxin receptor (Hartshorne and Catterall, 1981; Barchi and Murphy, 1981) with indications that it includes glycoproteins (Cohen and Barchi, 1980).

IV.B.2c. Variations in Toxin Structure

It would be interesting to establish whether there is a chiral preference at the receptor site. Both optical isomers of STX (1) are available from the total synthesis (Tanino *et al.*, 1977), although no resolution of the racemic mixture has yet been reported. The synthetic material has been evaluated (Pelhate and Sattelle, 1978) by electrophysiological studies on the cockroach giant axon, and was found to have the same properties as natural material, although the quantitation reported was not sufficiently precise to establish whether the racemic mixture was equipotent with natural material.

The most dramatic reductions in potency result from alterations at C-12. Kishi (1980) reported that synthetic 12-deoxo saxitoxin (19)

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was devoid of activity. C. Y. Kao *et al.* (1981), in electrophysiological studies on the squid axon, found αSTOH (13) to be 0.002 as potent as STX (1), and βSTOH (14) to be 0.001 as potent as STX (1).

Removal of the carbamoyl group, forming dcSTX (15), reduces the mouse toxicity to 0.67 that of STX (1), but a somewhat larger decrease, to about 0.1 that of STX, in the potency on isolated frog muscle (Walker and Kao, 1980).

Neosaxitoxin (7) has been found to be essentially equipotent with saxitoxin (1) *in vivo* (Figure 17), on isolated from muscle (Walker and Kao, 1980), and on squid axon at pH 6.50, 7.25 and 8.25 (the first pKa of 7 being 6.75). Titration studies by Rogers and Rapoport (1980), elaborating on earlier work by Schantz *et al.* (1981) showed that the acid dissociation constants of both α- and βSTOH (13 and 14) differed only slightly from those of STX (1). There are several examples in Figure 20 showing similarity of presumed charge and differences in potency or the converse, although these are all equivocal due to uncertainties about actual net charge and non specific binding *in vivo*. Taken together, however, and primarily on the strength of the data for 7, it appears that net charge can be excluded as a sole determinant of potency.

Shimizu *et al.* (1981) showed that dehydration of STX (1) to the ketone form (25) occurred only once the C-8 guanidinium had been deprotonated, while the results of P. N. Kao *et al.* (1981) indicate that it is the protonated form that is active, which in turn implies that the ketone form (25) is not. Both mouse bioassay (Koehn *et al.*, 1981) and

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electrophysiological experiments on squid axon (C. Y. Kao *et al.*, 1981) show βSTOH (14) to be less toxic than αSTOH (13). Yet Shimizu *et al.* (1981) found that the C-6/C-13 rotamer population of αSTOH (13) to resemble that of the inactive ketone form (25), while the rotamer population of the less toxic βSTOH (14) resembled that of the ketone hydrate (1).

Substitution of a sulfo group on the carbamate reduces *in vivo* potency much more than substitution of a hydroxysulfate on carbon 11. In the case of 1, the presence of the 21-sulfo group (forming 2) reduces *in vivo* potency by about 15X, while the similarly charged 11-hydroxysulfate derivatives 3 and 5 have potencies only slightly less than 1. From the lack of changes in H-6/H-13 coupling constants and relative H-13 shift positions, it does not appear that the carbamates and sulfamates differ greatly in rotamer populations. A similar synthetic derivative, decarbamoylsaxitoxin hemisuccinate (18) (Ghazarossian, 1977; Koehn *et al.*, 1981), has an *in vivo* potency of about 34 mouse units/mg, about 0.006 that of saxitoxin (1). The coupling constants and shifts again suggest little change in the rotamer population from that of saxitoxin.

Neither rotamer population nor net charge seem to correlate with potency, and the absence of the carbamate group reduces it only slightly, while the presence of an additional charged group on the carbamate (or hemisuccinate) side chain appears to have a more substantial effect. P. N. Kao *et al.* (1981) demonstrated that the charge on the C-8 guanidinium was essential to toxicity, the deprotonated form being inactive,

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and that charge on the C-2 side of the molecule was relatively unimportant. Of the 11-hydroxysulfate epimers, 3, which has the sulfate oriented alpha, toward the C-8 guanidino, is less potent *in vivo* than 5, which has it oriented away. Of the sulfamates, 4 is far less toxic than 6, and the potency ratio of 3 to 4 is far greater than that of 5 to 6. It seems entirely possible that the determining factor in each case is moderation of the charge of the C-8 guanidino, most complete in 4 where the 21-sulfo is complementary to the α-orientation of the 11-hydroxysulfate.

IV.B.2d. Competitive Interactions

Electrophysiological experiments by Hille (1968b) and Woodhull (1973) indicated the presence of a group with pKa about 5.2, the protonation of which blocked the sodium channel. Binding studies by Henderson *et al.* (1973, 1974) demonstrated competition between STX (1) and several cations. Competition with metal ions suggested that the site was also a metal binding site, probably negatively charged, while competition with protons suggested a binding site pKa of 5.6 to 5.9. The subsequent work of Spaulding (1980) demonstrated that the toxin binding site can be distinguished from the functional part of the channel, implying that there are at least two sites with negative charge.

Electrophysiological studies by D'Arrigo (1976) on crayfish axon, employed uranyl ion, which binds specifically to phosphate groups, and methylene blue, which binds strongly to carboxyl groups. Both were evaluated for their effectiveness in suppressing blockage by saxitoxin

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(1). His results indicate that the STX binding site probably involves carboxylate and not phosphate groups.

IV.B.3. Bioaccumulation

Some organisms accumulate large amounts of the toxins. The ability to bind the toxins would appear to play a significant role in this accumulation. Data are sparse for organisms other than those normally taken for human food, the most being available for bivalve molluscs.

The level of toxicity observed in a sample is a result of the rates of uptake and loss, complicated by the conversions among toxins of differing potency. The observed toxicity may include a substantial component from *Protogonyaulax* that have been ingested but not yet digested, reflecting the accumulation of dinoflagellates, rather than of toxins. Different species of bivalves under comparable circumstances differ substantially in the levels of toxicity they attain. Food selectivity may contribute to differences in rates of uptake (section III.C.1). The actual loss of toxins, distinct from a decrease in toxicity due to interconversions among toxins of differing potency, could result from either conversion to non-toxic substances or elution of bound material. While slow decreases in toxicity could therefore reflect a failure to degrade the toxins rather than more efficient retention, some degree of toxin binding is clearly indicated by the long term retention seen in the gills of *Mya arenaria* and *Spisula solidissima* (Prakash *et al.*, 1971), and in the siphons of *Saxidomus giganteus* (Pugsley, 1939; Quayle, 1969) and *Saxidomus nuttallii* (Sharpe, 1981).

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Since the toxins would be expected from *in vitro* experience to degrade at the pH and oxygen concentration to which shellfish are exposed and which likely prevail in much of their tissue, some sort of compartmentalization or other protective circumstance is possible, and may relate to the mechanism of retention.

The persistence of toxicity in butter clam (*Saxidomus giganteus*) siphons suggests that they might be rich in some constituent that binds the toxins. Quayle (1969), enlarging on the earlier studies by Pugsley (1939), demonstrated that toxicity was highest in the dark tip of the siphon and decreased toward the proximal end, as did pigmentation. This led Price and Lee (1971; 1972a,b) to suggest that the toxin might be bound by melanin. They found that melanin content and mouse assay results corresponded for three fractions obtained from toxic butter clam siphons. Using both natural and synthetic melanin and a chemical assay for STX (1) they evaluated STX-melanin binding with variations in pH and in the presence of various metal ions. Desorption of STX (1) increased with increasing acidity and increasing cation charge, Al^{+++} being the most effective. The results suggest that the binding to melanin is electrostatic and reversible, resembling the behavior of synthetic cation exchangers.

At least two toxins (9, 11) that have been frequently reported from shellfish (Shimizu, 1979) should bear no charge at likely *in vivo* pH. The recent detection of 4 and 6 in oysters (Onoue *et al.*, 1981) may also reflect retention of uncharged toxins by shellfish, although data in their report does not exclude the possibility that the toxins were within

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ingested *Protogonyaulax*. If, however, these observations do indeed reflect retention of uncharged toxins, they will obviously exclude mere ion exchange as the retention mechanism.

IV.B.4. Separations Chemistry

The repertoire of *in vitro* binding techniques pales when compared to the strong, specific, reversible binding accomplished by the natural substrates. There is obviously something to be learned.

It is useful to recognize that simplistic application of binding as a method for primary recovery of the toxins has proven treacherous. The group C toxins (section II.B.3) are not bound on the substrates commonly used. The group A and B toxins which may be bound, may also be lost from carboxylate resins in the hydrogen form due to low *in situ* pH or, in the case of 8, 9, and 11, from the ammonium form due to high *in situ* pH.

Binding to carboxylate resins appears, at the first approximation, to be electrostatic (section II.B.4). The elution sequence with acid is a function of charge, and the elution of 8, 9, and 11 with pH 7.5 buffer similarly implies a dependence on charge. It is not clear whether chromatographic fine structure is attributable to nuances of effective net charge, or whether other interactions are significant.

IV.C. Toxins and Food Webs

IV.C.1 The Perspective

As mentioned at the start, the phenomenon that we are familiar with as toxicity to humans from eating toxic bivalves is likely only the

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most apparent aspect of a general movement of toxins through food webs, various organisms differing in their susceptibility to the toxins and tendency to accumulate them. This may have a significant influence on predator/prey relationships, suppressing predation on accumulators except by insensitive consumers. Possible mechanisms are not limited to death from acute toxicity.

Toxins originating in *Gonyaulax tamarensis* were the apparent cause of a large mortality of seabirds along the northeast coast of England in 1968. Shags (*Phalacrocorax aristotelis*) apparently died as a direct result of eating the sand lance (*Ammodytes* sp.) which in turn were presumed to have obtained toxin from the zooplankters upon which they feed (Clark, 1968). It is tempting to speculate, on the basis of studies by White (1981b; see below), that fish accumulating relatively high levels of toxin were more susceptible to capture. On the other hand, mortalities among three species of terns (*Sterna* sp.) suggested less immediate effects, a large portion being females that died during egg-laying (Coulson *et al.*, 1968). White has documented the passage of toxins from *Gonyaulax excavata* to higher trophic levels (1979), resulting in recurrent kills of the Atlantic herring (*Clupea harengus harengus*) in the Bay of Fundy (1980, 1981a) and has shown in addition that pollock, flounder, salmon, and cod are quite sensitive to the toxins (1981b).

Whelks, carnivorous snails that prey on bivalves, are frequently found to be toxic (e.g., P. L. Goggins *in* Jensen, 1961) and have been shown to accumulate toxin from their prey (Ingham *et al.*, 1968; Caddy and Chandler, 1968).

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Greater susceptibility may account for the relatively low toxicities of oysters (Sribhibhadh, 1963; Neal, 1967) and soft shell clams (Prakash *et al.*, 1971) compared to other species under the same circumstances. Dupuy (1968) showed that pumping by oysters could be suppressed or stopped entirely by the presence of *Gonyaulax catenella*. Electrophysiological studies on nerves from various bivalves (Twarog *et al.*, 1972; Twarog and Yamaguchi, 1975) showed that those from soft shell clams and oysters were sensitive to the toxins, while those from scallops and mussels were quite insensitive.

At each step, accumulation of toxicity may involve either that of the toxins themselves, or merely of organisms containing the toxins. Given the high levels for the toxin content of *Protogonyaulax* cells found in the present study (section II.C.2), the toxicity of ingested, undigested material could account for high levels on a whole body basis, such that an organism need not actually retain toxins to be a significant source of toxicity to higher trophic levels.

Changes in composition likely accompany passage from one stage to the next, and occur within each stage. In *Protogonyaulax*, 1 seldom occurs unaltered (section II.C.4), and the 11α hydroxysulfate epimers are either absent or minor to their 11β epimers. In scallops from the Bay of Fundy, Fix Wichmann *et al.* have shown (1981a) that the epimer ratio is altered to that found *in vitro*, with the 11α epimers predominating, and that there is a loss of the N-1-hydroxyl. Shimizu and Yoshioka (1981), using scallops from the same area, demonstrated that digestion of a toxin mixture with scallop foot, viscera, or aductor

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muscle caused reductive removal of both the N-1-hydroxyl and 11-hydroxysulfate groups. In addition to these transformations, the separations properties of the toxins (section II.B and discussion in section III.B) suggest that the toxins will differ in the degree to which they are retained. Saxitoxin (1), the product of the transformations and the most strongly retained, would be expected as the eventual residue of such processes. Scallop muscle, however, was found (Shimizu and Yoshioka, 1981) to lower the overall toxicity of the mixture in a manner suggesting that the transformations can also be carried further, to non-toxic products and that detoxification may in part be an active process, rather than simple elution.

Although it should therefore be recognized that what appears to be retention might instead be a failure to degrade, apparent retention seems to differ greatly among organisms. Sribhibhadh (1963) and Neal (1967) found that mussels can increase abruptly in toxicity, attaining very high levels, but also lose toxicity fairly quickly. In contrast, butter clams (Neal, 1967; Quayle, 1969) become highly toxic and lose toxicity very slowly. Whichever mechanism accounts for the slow loss, high affinity or a failure to metabolize the toxins to non-toxic products, the low rate implies that a relatively low rate of input may suffice to maintain the relatively high levels of toxicity seen in butter clams.

Given the likelihood that marine food resources will become increasingly important to human well being, leading to novel patterns of utilization and dependence on optimal productivity, understanding the passage of the toxins through food webs will in the long term become

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important. The immediate need, however, is to deal with toxicity in bivalves. The following section describes some of the implications of the present work with respect to bivalve fisheries.

IV.C.2. Implications of the Present Work Relevant to Bivalve Fisheries, Particularly in Alaska

Sediments capable of giving rise to *Protogonyaulax* are widespread along the Alaskan coast. This does not address the possibility that entirely different source organisms occur, but does clearly establish, in accord with previous reports (Chang, 1971; Neal, 1967; Zimmerman and McMahon, 1976; Meyers and Hilliard, 1955) that the kind of organism generally accepted to be the toxin source can occur here.

Sediments rich in *Protogonyaulax* hypnozygote cysts may in themselves be a significant source of toxicity, as has been suggested for shellfish on the East coast (Bourne, 1965; Dale *et al.*, 1978; Yentsch and Mague, 1979). Cyst abundance appeared to vary widely (section II.A.4), being particularly high in the vicinity of Porpoise Island, which has a history of high shellfish toxicity. Since benthic cysts should be affected by the same processes that segregate other sedimentary material and produce non-uniform distributions over the bottom, and could be resuspended by wind and current action, they may help explain seasonal and spatial distributions of shellfish toxicity, particularly in butter clams, that have been difficult to reconcile with a phytoplankton source (Schantz and Magnussen, 1964; Neal, 1967).

It must be recognized, however, that none of the available data exclude the possibility that toxin is associated with sediment components

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other than cysts, nor is it clear that, given the refractory nature of hypnozygote cysts, such toxin as cysts contain would be available to shellfish consuming them. Still, both hypnozygotes and pellicle cysts are relevant to shellfish toxicity, each being a possible source of toxicity that does not resemble the motile cell and would not in the past surveys have been recognized as a source organism.

The circumstances of the estimates of toxicity and cyst abundance (section II.A.4) preclude detailed treatment, but the results do permit some useful guessing. The samples studied were taken from depths of 30 to 100 m and spaced such that they are probably a good representation for about 1 km^2 of bottom. The estimate of 200 cysts/mL probably applies to at least the top centimeter of most of this area. Extending the estimate of 0.2 mouse units per milliliter over this area and considering only the top centimeter gives an estimate of 2,000 mouse units per square meter of bottom, probably a lower limit.

Protogonyaulax hypnozygote abundance may prove to be a useful index for areas with a high risk of shellfish toxicity, being a possible source of both toxicity and of seed material for blooms (Anderson and Wall, 1978; Anderson and Morel, 1979). It will be particularly helpful if this proves to be the case, since benthic cyst abundance is likely to be a relatively conservative property, in contrast to the ephemeral nature of phytoplankton and water parameters, and would therefore need to be surveyed only once or twice a year to provide adequate warning of increased risk.

The toxicity of *Protogonyaulax* cells in nature is probably higher than has been appreciated, which may also help reconcile the disparity

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between the relatively low numbers of *Protogonyaulax* found in surveys that encountered fairly high levels of shellfish toxicity. If growth of *Protogonyaulax* in nature resembles growth in batch culture, the toxicity of cells probably varies greatly during the growth of a bloom, such that a simple correspondence between cell numbers and shellfish toxicity would not be expected.

Despite continuing efforts to develop a better method for determining toxicity, it is likely that bioassay will continue for some time to be an important tool for assessing toxicity risk. There are two problems with making reliable assessments, the first of which applies to bioassays. Preliminary tests of the conditions necessary for hydrolysis of the sulfamate toxins to the more toxic carbamates suggest that the conditions prescribed for sample preparation in the standard mouse bioassay (Association of Official Analytical Chemists, 1975) will not assure complete hydrolysis. If this hydrolysis occurs to a greater extent in product due to storage, processing, or digestion by the consumer, then the bioassay is not a safe estimate of risk. Increasing the acidity during sample preparation should solve the problem.

The second problem relates to both bioassays and chemical methods, all of which depend for their utility on a known relationship between the results obtained and risk to the consumer. Available estimates of human oral potencies are based on epidemiological surveys (e.g., Quayle, 1966; Prakash *et al.*, 1971) correlating the severity of human symptoms with the number of mouse units to toxin probably ingested. There is a great deal of scatter in the data, much likely attributable to different

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circumstances of ingestion, differences in human sensitivity, and the necessary imprecision of the estimates. The established quarantine limit of 80 micrograms saxitoxin equivalent per 100 grams shellfish meat, approximately twice the detection limit of the mouse bioassay, is derived from such data and has been thought to provide an ample margin of safety. If shellfish poison were only a single substance, or all had the same composition, or if the constituent toxins all had identical properties, or if the human oral and mouse ip potencies were on the same order, this might be so. None of these seem to be the case. Using extracts of toxic California mussels, early work (Prinzmetal *et al.*, 1932) established that in mice the ip potency was about forty times the oral potency and, with other animals, showed that the difference was not exceptional and appeared to be due to differences in absorption by the two routes. It is therefore reasonable to assume that a large, though undertermined, difference exists between ip absorption by the mouse and gastrointestinal absorption by humans. Given the differences in separation properties (section II.B) among the toxins it is clearly unsafe to assume that the ratio of human oral to mouse ip potencies is the same for all of them. With respect to bioassays, variations in the composition of shellfish poison therefore imply differences in the ratio between assay results and the risk to humans. For chemical methods, which may eventually permit quantification of the individual toxins, it will be important to recognize that the increment of consumer risk from each toxin may differ substantially from that apparent in the mouse bioassay.

The patterns of toxin composition among different strains, suggesting uniformity within but substantial differences among regions, imply

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that the nature of shellfish toxicity will show a similar regional variation due to the unique properties of each toxin. Management practices will therefore have to differ among regions but, fortunately, can be uniform within each.

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V. SUMMARY

1) Toxic dinoflagellates can be isolated from the Alaskan coast. All toxic strains fall clearly within the genus *Protogonyaulax*. No clear evidence was found for non-toxic organisms that would be assigned to *Protogonyaulax* by morphology.

2) Sediments that can give rise to these organisms, presumably containing *Protogonyaulax* hypnozygotes (resting cysts), are widespread.

3) At one location tested, sediment that appeared to have a high cyst concentration also proved to be toxic. Unless this sample is exceptional, there may be a significant reservoir of toxin in the sediment, which might be available to intertidal organisms through the action of waves and currents. Shellfish toxicity may therefore not be strictly dependent on phytoplankton growth.

4) Chemical studies of the toxins extracted from cultured *Protogonyaulax* revealed a novel family of toxins which had low acute toxicity until hydrolyzed to form the six toxins (1, 3, 5, 7, 9, 11) previously known. Four of the new toxins (4, 6, 10, 12) were found to crystallize. X-ray crystallographic analysis of 6 and 12, coupled with chemical and spectroscopic study of the entire group, showed them to be the carbamoyl-N-sulfo derivatives 2, 4, 6, 8, 10, and 12. Toxins from the new group occurred in most strains analyzed, generally at higher concentrations than the previously known toxins. The results suggest that the new compounds have possibly not been recognized in previous studies due to their low toxicity, facile hydrolysis, and altered chromatographic properties.

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5) Toxin content per cell, studied extensively in batch cultures of clone PI07, was found to vary substantially. Growth at lower temperature and phosphate concentration was found to give cells with higher toxicity. Since these conditions are closer to those found in Alaskan coastal waters than those that have been used in past culture studies, it appears that the toxicity of cells in the environment is likely to be much higher than has been appreciated. Toxin content per cell varied substantially during batch growth under most conditions tested. The reasons for these variations are not apparent, but they underscore first, the difficulty in assigning a value for the intrinsic toxigenicity of a clone and, second, the difficulty to be expected in correlating cell numbers with shellfish toxicity levels in nature. However, variations in toxicity among strains were in some cases large with respect to the variation seen within a strain such that some are clearly more toxic than others.

6) In contrast to toxicity, toxin composition of a strain changed little with growth conditions or stage of growth, suggesting that toxin composition was a relatively conservative property of a strain.

7) The patterns of composition in 10 strains isolated from San Francisco, Dutch Harbor, and points between suggested the existence of regions within which isolates had the same toxin composition and among which there were large differences. Five such regions were identified. On this basis it would appear that there are at least five distinct, uniform populations of *Protogonyaulax* in the northeast Pacific, emphasizing a need for a fundamental reassessment of the systematics of the

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group. Given the differing properties of the individual toxins, the patterns of composition also imply that, insofar as such organisms supply toxin to shellfish, the nature of shellfish toxicity will vary in a similar pattern.

8) While the *in vitro* chemistry of the toxins suggests that the state of the toxins *in vivo* somehow differs, the array of 12 toxins described appears necessary and sufficient to account for the observable characteristics of acute toxicity from *Protogonyaulax*.

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VI. EXPERIMENTAL SECTION

Water

Purified fresh water, designated 'QW', for all chemical and biological work was obtained from a Millipore Milli-RO4 unit combined with a Milli-Q water purification system which insured very low levels of organic or ionic contaminants. Seawater, designated 'SW', was obtained from the running seawater system at the Seward Marine Center, which draws from a depth of 70 m. Salinity of this water ranges from 31.5 to 33.5°/$_{oo}$, with a two year average 32.5°/$_{oo}$ (A. J. Paul, Seward Marine Center, personal communication).

Media

The media used in this study were based on seawater with Guillard's 'f/2' enrichment (Guillard and Ryther, 1962). The first (Beatrice Sweeney, personal communication), designated here as 'SG', is f/2 with silicate omitted and 10 mL soil extract added per liter. The second, designated here as 'f'', omits soil extract and is thus f/2 without silicate. Soil extract was prepared by heating University of Alaska/Fairbanks deciduous forest topsoil with an equal volume of seawater for several days, with occasional stirring. The amber supernatant extract was decanted and stored frozen. Media of nominal 25°/$_{oo}$ salinity (designated, e.g., 'SG25') were prepared by diluting 3 parts SW with 1 part QW prior to enrichment. Media in tubes and flasks were autoclaved in a 40-quart aluminum pressure cooker, heating until the pressure reached 15-20 psi and allowing to cool. Glass culture tubes (16 x 150 mm)

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with glass caps (chloridometer vials, 20 x 40 mm) were filled with ca 15 mL medium. Cotton-stoppered erlenmeyer flasks were filled to 40-50% of their volume.

Cotton-stoppered 20 L carboys received 15 L medium and were pasteurized in a vat of hot water for 1-2 h, bringing the internal temperature to 70-90°. Carboys generally included a 12 mm OD glass tube, sealed at the bottom, as a thermometer well, a 1/4" OD polypropylene air tube, and occasionally a 5/16" OD polypropylene tube for removing samples.

Culture Racks and Chambers

Tube cultures were held vertically in wire racks, receiving continuous illumination from the side at 30-40 microeinsteins m^{-2} sec^{-1}. Temperature in these tubes ranged from 10 to 20°C. Culture chambers were cooled by cold tap water running through an automotive radiator. Constant temperature was maintained by thermostat-controlled fans that increased air flow over the radiator as the temperature rose. Additional fans within each chamber operated continuously to improve temperature uniformity. Carboys in these chambers received about 50 microeinsteins m^{-2} sec^{-1} illumination from the side. Flask cultures were grown on open multi-level racks at room temperature (10-20°C), receiving about 20 microeinsteins m^{-2} sec^{-1} continuous illumination from above and twice that from below. Light measurements were made with a Li-Cor LI-185A meter (Lambda Instruments) with an LI-192S quantum sensor. Illumination was provided by banks of F40CW cool-white fluorescent lamps.

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Vat Cultures

Polyethylene vats, about 90 cm wide and 115 cm long, were lined with construction-grade 4 mil polyethylene sheet and filled with 500 L 0.45 micron Millipore filtered seawater. Autoclaved nutrients were added. The vats were illuminated from above by banks of fluorescent lamps, and were cooled by cold tap water running through coils of 1/2" OD polyethylene tubing immersed in the cultures. For the temperature study described below, temperature was maintained by manual adjustment of the amount of the coil immersed in a culture. For the remainder of the vat culture studies, the flow of water through each coil was controlled by a solenoid valve which in turn was regulated by a thermostat immersed in the culture.

Carbon dioxide was added to each vat directly through a fritted glass sparger, the rate of CO_2 addition being manually adjusted to maintain the desired pH. The pH of freshly mixed medium was close to that of seawater, so the cultures generally requires CO_2 only after several days growth.

Seawater and medium pH were measured with a combination electrode (Cambridge) and Chemtrix type 40E pH meter. The electrode was soaked in used buffer, then the meter standardized in fresh buffer, pH 8.00 at room temperature. The electrode was then allowed to equilibrate with seawater, while the samples, dipped from cultures or elsewhere, were allowed to reach room temperature. Once electrode response and sample temperature had stabilized, pH was determined for each sample.

Each vat was aerated with a single, open glass tube at a rate that produced a small boil on the surface. For sampling, the vats were

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stirred by increasing the air flow to a vigorous boil and moving the end of the tube around the bottom of the vat. Air for both the vats and carboys was provided from the lab low pressure air system, passed through a bed of activated charcoal.

Sediment Samples

Sediment samples were obtained by grab, dredge, corer, SCUBA, or scooped from the intertidal, and held refrigerated until diluted for incubation.

Phytoplankton Samples

Plankton tows were made with a 10 micron mesh net, 15 cm diameter x 1.5 m long (Eastside Net Shop, Bothel, Washington), the catch bottled and refrigerated, and examined within 48 h.

Sediment Incubations

Sediment samples, either whole or following sieving to concentrate the 20 to 90 micron fraction, were diluted with filtered seawater or enriched seawater medium and portioned into 96-well "Microtiter" polystyrene plates (Cooke Engineering). Sediment was generally diluted 1:3 with medium in a 4 mL vial, 2/3 of this portioned into the first row of 12 wells, the remaining 1/3 diluted again with medium and the sequence continued for the remaining seven rows. This provided a broad range of sediment:medium ratios. The polystyrene plates had either been aged (stored for several years following manufacture) or conditioned in

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seawater and air-dried for 1 week. The filled plates were stacked and placed in polyethylene bags on illuminated culture racks (30-50 micro-einsteins m^{-2} sec^{-1}) at room temperature, about 15°C.

To evaluate the importance of light and temperature in germination, replicate plates from one sediment sample from Porpoise Island were diluted as usual but placed either in the dark, under an aluminum foil cover at room temperature, or returned to the refrigerator (about 4°C).

To quantify the number of cysts ready to germinate, a sample was taken by Shipek grab at a depth of 40 m just east of Porpoise Island and sieved to concentrate the 20-93 micron fraction. The concentrate was then diluted by factors of ten, portioning all 9 mL of each dilution into about 50 wells of a 96-well plate. These were incubated as above, scanned under a dissecting microscope, and a tally kept of the number of wells that gave rise to motile dinoflagellates resembling *Protogonyaulax*. A portion of the concentrate was extracted with HCl for mouse bioassay.

Pipet Isolations

Dinoflagellate cells, either from plate incubations or from phyto-plankton samples, were isolated by micropipet under a dissecting micro-scope. Due to the sediment in the wells, cells in the multiwell plates were most easily found when illuminated from both above and below. Cells harvested from a plate were often pooled, so it is generally not known, when more than one isolate came from a plate, whether they came from the same well and may therefore be sympatric or came from separate wells and therefore different cysts. The unit isolated — a single cell

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or chain of two cells — was washed at least twice in sterile medium (autoclaved or 0.22 micron Millipore filtered) before transfer to sterile medium in a glass tube or multiwell (24 well, Costar) polystyrene plate. The parent cell for clone PI07 was carried through 9 washes and transferred directly to a tube of sterile medium. Multiwell plates were placed on illuminated culture racks (30 to 50 microeinsteins m^{-2} sec^{-1}) at room temperature (about 15°C) in polyethylene bags, along with a piece of moist paper to further reduce evaporation.

Stock and Production Cultures

Stock cultures were maintained in tubes of medium SG25. Trials indicated that cultures were more tolerant of salinities lower than 33 than above 33°/$_{oo}$, and accepted 25°/$_{oo}$ well. It was thus expedient, for cultures that might be held for several weeks, to formulate media at the lower salinity so that, despite evaporation, salinity remained in an acceptable range. Maintenance transfers were made at intervals of two weeks to two months, the latter being acceptable but approaching a safe upper limit. For production of biomass, additional tubes were innoculated from the main stock line and, when they were growing vigorously, poured into flasks. Tube transfers were made with dry-sterilized cotton-plugged Pasteur pipets in a separate room, but not in a hood or with other sterile techniques. Transfers at other stages were made simply by pouring.

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Mouse Bioassay

Determinations of toxicity were based on the AOAC mouse bioassay. Male Swiss-Webster mice (Simonsen Laboratories, Gilroy, California) weighing 17 to 23 g received intraperitoneal injections of 1.0 mL of the test solution at a pH between 2 and 3. The mouse was observed until it expired with standard symptoms and the time noted from injection to the last gasping breath (generally the last of a series of about 3 to 5 shallow thoracic movements of decreasing magnitude). The dilution of the test solution was adjusted (with water which had been acidified to pH 2-3 with HCl) for each successive mouse, aiming for a death time of 6 minutes. Using the mouse weight and death time, the number of mouse units was obtained from Sommer's table (Sommer and Meyer, 1937; McFarren, 1971) and multiplied by the dilution factor to obtain the potency of the original solution. A minimum of three such values, excluding those for mice that survived more than 15 minutes or died in less than 4 minutes, were averaged to obtain the final potency value. Note that this differs slightly from the AOAC method, which uses the median value of three mice, acceptable if the median death time falls between 5 and 7 minutes. Mice outside the weight limits were used only for approximate assays. Mice that had survived previous assays were used only to approximate the potency of solutions known to be lethal.

For standardization, vial #421, lot 7 FDA Standard Shellfish Poison was opened and 0.100 mL aliquots transferred to tared 4 mL vials containing about 0.5 mL 0.1 M HCl. These aliquots were stored frozen. The sequence was repeated several months later with vial #642, lot 8

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FDA Standard Shellfish Poison. The FDA standard is stated to contain 100 micrograms/mL shellfish poison, which is defined to have a potency of 5500 mouse units per milligram. For use, a vial was thawed, diluted with water, and weighed to determine concentration. Dilutions of this were then made and injected as for regular samples. Death times, mouse weights, dilution factors, and the concentration of the aliquot of standard were used to calculate the number of mouse units per milligram of standard shellfish poison. The average of 50 such standardizations over an 18 month period, using both batches of aliquots, each standardization based on 3 to 5 mice, was 4342 mouse units per milligram with a standard deviation of 506.9 (11.67% of the mean). As a result, all toxicities reported here have been adjusted by a factor of 5500/4342 = 1.267.

Toxicity of Isolates

To survey isolates for toxicity, cultures were grown to volumes ranging from 200 mL to several liters. The cells were counted and a portion removed, centrifuged, and the packed cells washed with filtered seawater to a small tube. This was again centrifuged, the supernatant removed, and the packed cells extracted with dilute HCl on a 100°C bath for 5 to 15 minutes. The supernatant was removed, its pH adjusted with dilute aqueous NaOH to between 2 and 3, and 1.0 mL injected into a mouse. The quantities were chosen such that toxicity over about 5 micromouse-units per cell would give a strong positive. In most cases, the mouse either died in less than two minutes or survived.

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Preparation of Thecae for Microscopy

The method is a simplification of that described by Adamich and Sweeney (1976) for the preparation of *Gonyaulax polyedra* spheroplasts. *Protogonyaulax* cells were centrifuged from an actively-growing culture and resuspended in QW. After standing 15 minutes, a detergent solution (Alconox) was added to about 0.2% weight/volume. After 10 minutes, this suspension was centrifuged, the supernatant removed, and the pellet resuspended in 0.2% Alconox. After 10 minutes further standing, the suspension was again centrifuged and the supernatant discarded. Over 50% of the resulting pellet was clean, empty, intact thecae.

Cell Counts

Cells were counted live under a dissecting microscope by scanning a sample contained in a flat glass capillary. For calibration, sections of capillary (Vitro Dynamics, Rockway, NJ) about 30 cm long were weighed dry, after filling with water for a measured length, and after emptying. The difference between dry and emptied weights was found to be negligible for these purposes. The filled length and weight of contained water were used to calculate the mean cross-sectional area of each tube. On the basis of this calibration, the tubes were marked at lengths corresponding to convenient multipliers, e.g. 5X, 10X, to calculate the number of organisms per milliliter from the number contained in a marked segment. For this method to work, the following conditions must be met: 1) the scanning rate along the tube must be fast compared to the swimming rate of the organisms; 2) the depth and width of the lumen must be

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smaller than the depth and width of the microscope field, such that the entire lumen is continuously observed; 3) the magnification must be high enough to permit recognition of the organisms counted; 4) the density of cells in the field must be low enough that the observer can keep track of which cells have been counted; 5) both dimensions of the lumen must be large enough to insure unobstructed passage of cells into the capillary; 6) the number of objects counted, and thus the volume, must be sufficient to insure that the number obtained is a satisfactory estimate of the value for the population sampled. For *Protogonyaulax* cultures in the range of 500 to 10,000 cells/mL, these constraints are satisfied by capillaries 0.2 x 2.0 mm and 0.3 x 3.0 mm internal cross-section. Lower densities were counted by summing the results of multiple samples with the larger capillary. For higher densities, capillaries with a square cross-section, e.g. 0.3 x 0.3 mm, proved optimal. For counts, the sample container or culture flask was mixed, the capillary dipped in the suspension, and sample aspirated by mouth long enough to insure that several volumes of sample passed through the tube. Aspiration was stopped and the tube lifted clear of the sample simultaneously. The surface of the capillary was wiped dry. The sample was scanned immediately under the dissecting microscope, covering a marked section chosen such that 100 to 400 objects were counted. Single cells and multiples were tallied separately. Three such counts were made, the total number of cells calculated for each, and the three numbers averaged to determine the final value. The three counts usually took less than 15 minutes, with the standard deviation for the three generally being less than 10% of the mean.

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Packed Cell Volume

Cells for packed volume and toxicity determinations were concentrated by preliminary centrifugation, resuspended in filtered SW, and transferred to thrombocytocrit tubes (Thomas). Since these are no longer listed in the Thomas catalog, some description is in order. The upper section of the tube is an uncalibrated bulb, ca 7 mL volume. The lower section is an open capillary, calibrated from 0 to 0.03 mL with 0.5 microliter graduations. The flat-ground bottom of the capillary is closed by a spring-mounted metal cap with a rubber seal. Prior to use, the thrombocytocrit was rinsed and the capillary portion filled with filtered SW. The sample was added and the assembly centrifuged for 5 minutes at 2000 rpm in an IEC model HN desk-top centrifuge at room temperature. Based on cell counts, the original sample volume was adjusted to give about 20 microliters packed cells in each tube. Cell volumes were read, the supernatant aspirated, the closure removed, and the packed cells extruded from the capillary into 0.2 M HCl.

Proctor Enhancement

To evaluate conditions for preparing samples for assay, two 1650 mL aliquots were removed from a vat culture of clone PI07, centrifuged, and the supernatant medium removed. Cells from the first portion were resuspended in 5 mL 0.1 M HCl. Three 1.0 mL aliquots of this suspension were heated in a 100°C bath for 1, 5, and 25 minutes, a fourth aliquot not being heated. Cells from the second portion were resuspended in 5 mL 0.01 M HCl. Three 1.0 mL aliquots of this suspension were removed, the

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second being adjusted to 0.09 M and the third to 1.1 M in HCl. The three tubes were heated for 10 minutes in a 100°C bath. In both cases the tubes were cooled, centrifuged, supernatants removed, cells washed two more times, and the combined supernatants from each tube assayed.

Dilutions for assay ranged from 30 to 160X, the pH of the injected solution being in the range 2-3. Adjustment, when required, was by varying the acidity (with HCl) of the diluent water.

Sample Preparation for Quantitative Assays

For routine quantitative assay of toxicity in cultured cells, the packed cells in replicate thrombocytocrits were extruded into a tube containing ca 1 mL 0.2 M HCl. This suspension was heated for 5 minutes in a 100° water bath, allowed to cool, the volume determined, and a portion of the clear supernatant removed for assay. Samples were often stored refrigerated for several days after they were acidified. Bulk extracts, chromatographic fractions, and purified toxins were assayed either without further treatment or following 5 minutes heating in a 100°C water bath of 0.5 to 5 mL solution that had been acidified to 0.1 to 0.4 M with HCl. The higher concentration of acid was employed for samples suspected to have substantial buffer capacity, to insure that the hydrogen ion concentration was 0.1 M or greater. In samples of low potency where dilution for assay would not suffice to bring the pH of the injected solution within the range of pH 2-3, the pH was adjusted with aqueous NaOH following digestion. For chromatographic fractions this was most easily accomplished by making up stock solutions of

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ca 4 M HCl and 3 M NaOH, adjusted such that addition of, e.g., 0.1 mL acid before heating and 0.1 mL base after cooling gave the desired pH at each step.

Preliminary Carboy Experiments

Three carboys were prepared with normal f' medium, f' with the nitrate enrichment omitted, and f' with the phosphate omitted. Each was innoculated with 1.0 L culture from a carboy of PI07 grown in f' medium, giving an initial concentration of about 500 cells/mL. The carboys were maintained in a growth chamber at about 10°C, receiving about 50 micreinsteins m^{-2} sec^{-1} continuous cool-white fluorescent illumination. Samples were taken for toxicity determinations after 12 and 27 days of growth.

Specific Conditions for Vat Culture Experiments

In each of the following six series, a single parameter was adjusted to a different level in each of four vat cultures grown and sampled simultaneously. For sampling, the vats were stirred by turning up the flow in the aeration tube and moving it around the bottom of the vat. With the air tube then placed in the center of the vat, two samples were dipped from ca 5 cm below the surface with two polypropylene centrifuge bottles (Corning 25350, nominal volume 250 mL), one from the center of the boil and the other from ca 30 cm away to confirm that the sample was well mixed. Each bottle was counted three times as described above, and the mean of the two samples accepted as the cell concentration for

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the vat. The two values generally agreed within less than 10%, most often within less than 5%. From the counts, volumes estimated to yield about 20 microliters packed cell volume were transferred to a second set of centrifuge bottles and centrifuged 5 minutes at 1500 g in an IEC model K at room temperature. The supernatant was aspirated and the cells washed from each to a thrombocytocrit and centrifuged to determine packed cell volume. The cells from both thrombocytocrits were extruded into a single tube for assay as noted above. Sampling thus gave replicate counts and packed cell volumes, each pair being used to calculate volume per cell, and a single determination of toxin content which was used with the averages to calculate toxin content per liter of culture, per cell, and per microliter of packed cells. To some extent, this permitted internal cross-checking to appraise trends or disparate values.

Aeration: A preliminary series of vats was grown at levels of aeration substantially above and below that normally used. These were monitored for growth, but not for toxicity.

Temperature: Nutrients were added to f' enrichment with 50% of the normal phosphate, the pH maintained at 8.4 ± 0.4 with added carbon dioxide. Illumination was adjusted to 50-75 microeinsteins m^{-2} sec^{-1} at the surface center. Each vat was innoculated with one carboy of PI07, all grown at about 10°C. The vat temperature was regulated by manually adjusting the degree to which the cooling coils were immersed and held within 0.5°C of the nominal settings 6, 8, 10, and 12°C.

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pH: Nutrients were added to f' enrichment with 50% of the normal medium phosphate and the vats each innoculated with a carboy of PI07 grown at about 10°C. Illumination was adjusted to 70-80 microeinsteins m^{-2} sec^{-1} at the surface center. The pH was maintained at the nominal levels of 7.7, 8.0, 8.4, and 8.8 by adjusting the rate of carbon dioxide addition. Since the variation of pH from nominal was large with respect to the interval, the complete record is shown in Figure 22c.

Light: Nutrients were added to f' enrichment, the vats each innoculated with a carboy of PI07 grown at about 13°C, and pH maintained at 8.2 ± 0.2 by the addition of carbon dioxide. Temperature was maintained at 9.0 ± 0.9°C, except for the vat at highest light, which averaged about 0.3°C higher. Due to an irregularity in the water system, all four cultures experienced a brief increase to about 11°C on day 17, returning to normal within 12 h. This had no apparent effect on culture growth or toxicity. Illumination of the four vats was varied by adjusting the height of the normal 4-tube light and by adding 2 lamps to the third and four lamps to the fourth. Light intensity was measured both at the center and at the corners and midpoints of the sides. Considering the surface of each vat in quadrants, a value for "mean surface illumination" was obtained by averaging the corners of each quadrant and taking the mean of the four averages. In summary:

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Vat	Total lamps	Lamp height above surface (m)	Surface intensity ($\mu E \cdot m^{-2} \cdot sec^{-1}$)	
			center maximum	Mean
1	4	96	27	23
2	4	64	50	39
3	6	38	120	79
4	8	25	190	127

Phosphate: Nutrients were added to f' enrichment, with phosphate reduced to 0, 10, 20, and 40% of normal, and the vats each innoculated with a carboy grown at 12 to 13°C. Illumination was adjusted to 85-90 microeinsteins m^{-2} sec^{-1} maximum at the surface and pH maintained at 8.14 ± 0.20 by the addition of carbon dioxide. Temperature was maintained at 8.5 ± 0.5°C.

Nitrate: Nutrients were added to f' enrichment with 10% of the normal medium phosphate and nitrate altered to 1/4, 1, 4, and 8x the normal. The fourth vat was started at normal and increased to 8x on day 4. The vats were each innoculated with a carboy grown at about 12°C, illuminated at 80-100 microeinsteins m^{-2} sec^{-1} surface maximum, and the pH maintained at 8.4 ± 0.4 by the addition of carbon dioxide. Temperature was maintained at 8.5 ± 0.5°C with the exception of the first vat, which experienced transient decreases to about 7°C on days 6 and 12. These had no apparent effect on growth or toxicity of the culture.

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Vat Harvest

Vat cultures were harvested by pouring through a conventional 10 cm x 2 m 10 micron-mesh phytoplankton net. One pass through the net recovered at least 90% of the cells and took between 15 minutes and 1 h, depending on the condition of the culture. The concentrated slurry of cells was centrifuged in 250 mL polypropylene bottles and extracted with dilute acid.

Column Chromatography

Columns were fabricated from 1" OD heavy wall Pyrex tubing or from 54 mm OD standard wall Pyrex tubing reduced at one end and joined to a short section of the 1" stock. The columns were found to have internal cross sections of ca 2.4 and 19.3 cm^2, respectively.

The columns were plumbed with 1/8" OD polyethylene tubing (Bel-Art), bore either 0.22 or 0.42". These were joined with short sections of #22 or #18 stainless steel hypodermic needle stock, which forms a leak-tight slip fit.

Lower end fittings were fabricated from 1" nylon end caps (Swagelok NY-1610-C) machined and joined to 1/8" nylon end caps (Swagelok NY-200-C) with either cyanoacrylate glue ("Super Glue") or with liquified phenol. Both formed a strong bond that tended to fail after several months use.

Bed supports were cut from 20 micron Nitex nylon mesh. Washers cut from Parafilm were used to seal and fill dead space between the end face of the column wall, the bed support, and the end fitting. The upper fittings at the columns were made from rubber stoppers penetrated with

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either #22 or #18 needle stock. Eluant was supplied by a peristaltic pump (Technicon Autoanalyzer Pump II) in which the tubing bore was selected to supply the desired flow rate. Gradients were formed by pumping limit eluant into a graduated cylinder that contained starting eluant and was mixed by a continuous stream of nitrogen. Drawing mixed eluant from the cylinder at twice the rate that limit eluant is pumped in results in a linear gradient. The columns were calibrated by determining the weight of contained water for measured segments to determine the mean cross-sectional area for each column. Bed height for each run was measured to calculate the bed volume for that run. Due to changes in the vinyl pump tubes with use, the flow rate was determined for each run, generally both at the beginning and end of the run. Fractions were collected by time, with the interval similarly calibrated for each run, although the interval proved to be quite stable compared to the variations in flow rate.

The columns were slurry packed under gravity flow. BioGel P2 (Bio-Rad Laboratories) polyacrylamide gel, 200-400 wet mesh, was slurried with water and allowed to hydrate for several hours. Sephadex G-10 (Pharmacia) 40-120 micron hydrated diameter, was also slurried with water and allowed to hydrate for several hours. Amberlite IRP64 (Rohm and Haas) 100-400 mesh, carboxylate cation exchange resin in H^+ form was hydrated and cycled from H^+ to Na^+ form and back several times, using HCl and NaOH. Fines were decanted at each step and the resin left in the hydrogen form.

Conductivity in the effluent was monitored with a Chemtrix Type 70 conductivity meter, the electrodes of which were connected by clip-leads

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to a multi-position switch. Conductivity cells were made from segments of steel needle stock, #22 or #18 as needed, connected by a piece of 1/8" OD polyethylene tubing such that there was a small gap between them. The gap, on the order of 1 mm, was adjusted to provide a convenient cell constant. Cells were connected to the multi-position switch by clip leads. Capacitance error in the system was detectable, but small compared to the signal.

In some runs, the pH of the effluent was monitored with a Benchmark 737 (Markson) flow-through electrode placed downstream from the conductivity cell. The pH electrode had a 0.85 mL dead volume and was therefore not generally used on analytical runs.

Total residue in column fractions was determined approximately by spotting 10 microliter aliquots of each fraction on a piece of polyethylene sheet and drying at ca 60°C. Observation of the residue under a dissecting microscope gave some indication of the kind and amount of material eluting.

Toxic fractions were located after the method of Buckley *et al.* (1976). Five microliter aliquots of each fraction were applied to sheets of E. Merck Silica Gel 60 F254 precoated TLC plates, air dried, and observed under both 366 nm and 254 nm UV light to locate fluorescent or UV-quenching spots. The plates were then sprayed with 1% hydrogen peroxide, heated for 15 minutes at 120°C, and again inspected under both wavelengths of UV. Toxic fractions produce blue or yellow fluorescing spots under these conditions. Aliquots (5 microliters) of the fractions producing fluorescent spots were applied to the lower margin

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of aluminum-backed E. Merck Silica Gel 60 F254 precoated TLC sheets, along with mixtures of standard toxins, and allowed to air dry. These were developed in pyridine:ethyl acetate:acetic acid:water (15:5:3:5) for 3 h, dried on a warm (ca 50°C) surface, checked for fluorescence, and then visualized as above. The resulting chromatograms were photographed under hand-held 366 nm UV illumination using a Wrattan 2A or 2E filter on the camera.

Analyses for Toxin Composition

Cell Harvest: Dinoflagellate cultures, in either vats or carboys, were stirred and aliquots removed for counts. A measured aliquot of the culture (20 to 100 liters) was then poured through a 10 micron plankton net into a polyethylene barrel. The concentrated slurry of cells was transferred to a 250 mL polypropylene centrifuge bottle and the net rinsed by passing the filtrate through it two more times, transferring cell slurry to the bottle each time. Cell recovery by this sequence is well over 90%. The resulting slurry was centrifuged briefly and the supernatant decanted to a tared bottle. The centrifuging was done at room temperature (about 20°C) in an IEC model K, but was done quickly enough that the cells, which had generally been grown at about 10°C, remained substantially below room temperature until the supernatant had been decanted.

Extraction: The cells were then extracted, unless otherwise noted, at room temperature with 5 washes of 1 M aqueous acetic acid of about 3 volumes each. The mixture of cells and acid was allowed to stand for several minutes after each wash before centrifuging. The combined

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supernatants were frozen and lyophilized with a few drops of n-octanol added to suppress foaming.

Assay of Seawater Supernatant and Cell Pulp: In some cases the residual pulp of extracted cells was digested with HCl (about 0.5 M, 15 minutes, 100°C). A portion of the seawater supernatant from the initial centrifugation was acidified to 0.2 to 0.5 M with HCl and heated for 5 minutes at 100°C. These solutions were assayed for toxicity, following adjustment of pH as necessary, to estimate the amount of toxicity lost to the supernatant or not recovered from the cells during extraction for chromatography.

Drying and Resuspension: The dried extract was taken up in QW, transferred to a tared 60 mL serum bottle, and again lyophylized. The residue weight was determined and water added to ca 0.3 g solids/mL, giving a slightly viscous suspension, pH about 4.5. Aliquots (0.10 and 1.0 mL) were removed from the stirred suspension for assay and chromatography. The solids content of these aliquots was estimated from the total suspension weight using a nomograph constructed from trials with a large batch of extract.

Chromatography: A column of BioGel P2 (bed about 2.4 cm^2 cross-section, 115 to 120 cm high) was washed for at least 1 bed volume, either initially or following the previous run, with 0.1 M acetic acid (about 14 mL/hour). Column parameters were determined precisely for each run. Flow was interrupted, the eluant run down to expose the top of the bed, and 1.0 mL extract applied. The extract was run on to the column under gravity flow, collecting the effluent to include in calculating elution

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volume. Under continued gravity flow, the top of the column was rinsed and the top surface of the bed stirred as necessary to prevent blockage from particulates in the extract. Once uniform flow was assured, the column was plumbed back to the pump. Fractions were collected at 12 minute intervals, starting before the void volume eluted, generally about 30% bed volume.

Comparison of Extraction Methods: Six 48 L aliquots were removed from a vat of PI07, each filtered and centrifuged. The ca 8 mL packed cells from each aliquot were extracted by one of the following methods: a) 1 M aqueous acetic acid, as described above; b) cells were slurried with 2 volumes of 0.1 M acetic acid, frozen, thawed, and extracted 5 times with a total of 91 mL 0.1 M acetic acid. The extract was lyophylized; c) cells extracted 6 times with a total of 89 mL 0.05 M aqueous HCl. The lyophylized extract was taken up in water to give a suspension with a pH of about 2. This was adjusted with ammonium acetate buffer to about 2.5 prior to chromatography; d) cells extracted with 6 washes of methanol, 0.5 M in acetic acid; e) cells extracted with 6 washes of 80% aqueous ethanol, 0.5 M in acetic acid; f) cells rinsed to a beaker with 200 mL 80% ethanol, 0.01 M in HCl, boiled for 15 mintues, centrifuged, and the cells washed twice more with 100 mL portions of the same solvent mix. In d, e, and f alcohol was removed from the extract under vacuum at room temperature, the aqueous residue washed with a minimum 3 x 15 mL chloroform, and the combined chloroform layers backwashed with water. The combined aqueous extracts from each were lyophilized and handled as normal extracts for chromatography.

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Conversions

To evaluate the conversions responsible for Proctor enhancement, solutions of toxins 2, 4, 6, 8, 10, and 12 that were 0.1 to 0.4 M in HCl were heated 5 minutes in a 100°C bath, cooled, lyophilized, and taken up in QW. These were compared by TLC with samples of the twelve toxins.

Purification of Toxins

The freeze-dried extracts from vat cultures of PI07, 5-15 g dry solids, were taken up in QW to about 0.3 g/mL and applied to large (19.3 cm^2 x about 100 cm bed) columns of BioGel P2 operated in a manner analogous to those used for analytical chromatography. On the basis of TLC, fractions of similar composition were pooled and freeze-dried. Several of the extracts utilized in the preparative work had been obtained at early stages in the study and had been exposed to high concentrations of HCl and other abuse. The pooled fractions containing toxin groups A, B, and C, separately or in combination depending on the resolution achieved in the first step, were applied to a short (2.4 cm^2 x about 30 cm bed) column of IRP 64 in the H^+ form, preceeded by the application of 10 mL 1 M ammonium hydroxide with a brief water wash. Elution of the column with water removed the group C toxins. Step or gradient increases in eluant acidity up to 1 M acetic acid removed first group B, then A, well separated but with little resolution within the groups. The three groups were again lyophilized. When it was judged necessary, particularly in the drying of group C at this step, solutions received a small amount of pyridine or ammonium acetate as a buffer prior to freeze drying. The group A toxins (1 and 7) were resolved on a column of IRP64 in the

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hydrogen form (2.4 cm^2 with a gradient of acetic acid from 0.1 to 1.0 M, flow rate about 50 mL/h, alternated with isocratic elution from BioGel P2 (19.3 cm^2 x about 100 cm bed) with 0.1 M acetic acid (about 100 mL/h).

Group B toxins were resolved by a combination of the following: a) isocratic 0.1 M acetic acid elution from Sephadex G-10 (2.4 cm^2 x 120 cm); b) application to a water-washed Pasteur-pipet microcolumn (bed volume about 1 mL) of IRP64 in the ammonium form, followed by elution first with 0.1 M ammonium acetate, pH 7.5, then with 0.1 M acetic acid; and c) gradient elution from IRP64 in the hydrogen form (2.4 cm^2 x 120 cm), generally with a preload of 10 mL 1 M ammonium hydroxide followed by a brief water wash. Flow rates were about 50 mL/h, gradients were generally linear and were within the range 0 to 0.1 M acetic acid. d) isocratic 0.1 M acetic acid elution from BioGel P2, either 2.4 cm^2 x 120 cm, 15 mL/h, or 19.3 cm^2 x 100 cm, 100 mL/h.

The various steps were repeated for group A and B toxins until satisfactory purification was achieved.

The group C toxins were taken up in QW and applied to a water-washed column (19.3 cm^2 x 110 cm bed) of BioGel P2 and eluted with water at about 100 mL/h, collecting 12 minute fractions. Fractions were evaluated by TLC, pooled into those containing $\underline{4}$ or $\underline{6}$, and freeze dried. The $\underline{6}$-group was taken up in warm water, about 50-55°C. Crystals of $\underline{6}$ formed on cooling and were recrystallized from warm water. In some preparations the initial crystallization of $\underline{6}$ gave also long, slender spicules that proved to be guanosine (IR, ^{1}H-NMR, melting point) but were easily segregated by washing. The $\underline{4}$-group was taken up in QW (about 100 mg/mL) and $\underline{4}$

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crystallized by the addition of methanol until the solution began to cloud, warming to clear, and allowing to cool. The mother liquors from such crystallizations were combined, lyophilized, applied to a BioGel P2 column (2.4 cm^2 x 120 cm) and eluted with 0.1 M acetic acid. Fractions rich (TLC) in $\underline{\underline{10}}$ and $\underline{\underline{12}}$ were lyophilized and applied to a similar column that had been equilibrated with 0.1 M ammonium acetate buffer, pH 8, and eluted at pH 8, resolving $\underline{\underline{10}}$ and $\underline{\underline{12}}$ from $\underline{4}$ and $\underline{6}$. Toxins $\underline{\underline{10}}$ and $\underline{\underline{12}}$ were then resolved by chromatography on a similar column, equilibrated and eluted with water. Toxin $\underline{\underline{10}}$ was crystallized from ethanol/water mixtures in a manner analogous to that used for $\underline{4}$. Toxin $\underline{\underline{12}}$ was crystallized by the evaporation of an aqueous solution.

Standard Solutions

As the final step in purification, toxins $\underline{1}$, $\underline{2}$, $\underline{3}$, $\underline{5}$, $\underline{7}$, $\underline{9}$, and $\underline{\underline{11}}$ were bound to IRP64 in the hydrogen form (2.4 cm^2, 50 cm bed), washed with QW and eluted with dilute acetic acid to insure that acetate was the counterion. The resulting toxin acetates were lyophilized and weighed. These and preparations of $\underline{4}$ and $\underline{6}$ that had been recrystallized, lyophilized, and weighed were taken up in QW to give 20 mM stock solutions.

Specific Potencies

Samples of $\underline{1}$-$\underline{\underline{8}}$, prepared as above, were taken up in QW and aliquots removed for assay. The weight of freeze-dried solids in each sample was determined either before dissolution, after removal of the aliquots, or,

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most commonly, both, and was generally determined for the entire batch of toxin (20 to 200 mg), to minimize weighing errors. The aliquots were assayed either directly or after addition of HCl to 0.2 M and heating for 5 minutes in a 100°C.

NMR Spectroscopy

^{1}H-NMR and ^{13}C-NMR spectra were obtained through collaboration with Dr. Heinrich K. Schnoes and with the assistance of Carol Fix Wichmann at the Department of Biochemistry, University of Wisconsin. Solutions of purified toxins were filtered into 5 mm tubes and lyophilized. For ^{1}H-NMR, the tubes were capped with septa, dried further through a needle, and redissolved in D_2O (BioRad, 100%). Chloroform was added as reference, with the assigned value δ = 7.27, and spectra run on a 270 MHz Bruker FT-NMR spectrometer. For ^{13}C-NMR, the samples were redissolved in D_2O with dioxane (δ = 67.6) as reference and spectra taken at 50 MHz on a JOEL XL-200 FT-NMR spectrometer with the exception of compound 8, for which spectra were taken in a 12 mm tube on a Nicolet NT-200 FT-NMR spectrometer. For ^{1}H-NMR spectra in DMSO, septum-capped tubes of dried toxins 3, 4, 5, and 6 were further dried at room temperature for 12-24 hours at about 10^{-2} microns. Aldrich "100.0%" $^{2}H_6$-DMSO, dried by passage through a bed of 4Å molecular sieve at 100°C with N_2 carrier gas, was introduced through the septum. TMS was added for reference.

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Elemental Analysis

Combustion analysis was performed by Galbraith Laboratories, Knoxville, on crystallized 6 and on purified 8 acetate. Both preparations were lyophilized at 10 microns prior to analysis.

Water Loss

To determine the weight loss associated with the transition from crystalline 6 to the amorphous, dried material used for elemental analysis, several ca 10 mg portions of freshly-prepared crystalline 6 were blotted dry on filter paper, transferred to tared aluminum weighing boats, and weighed on a Cahn balance. Inspection of these under a stereomicroscope indicated that mother liquor had been removed and that the material was still crystalline. The samples were then dried (room temperature, 10 microns) to constant weight.

Electrophoresis

Paper electrophoresis was performed by Mr. Frank Koehn, Department of Biochemistry, University of Wisconsin. Samples were applied to Whatman #1 paper, dried, and run at 1200 V for 45 minutes in pH 4.5 sodium acetate buffer.

X-ray Crystallography

X-ray diffraction studies of crystalline 4, 6, and 12 were conducted by Dr. Stephen D. Darling at the University of Akron. Single crystals of 6 were mounted in Lindemann capillaries with traces of mother liquor to

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prevent disintegration. The structure was solved by direct methods using 1764 reflections collected by the θ-2θ scan technique at room temperature using graphite monochromated Mo Kα radiation. Heavy atoms were located and the data refined using programs MULTAN 74, CRYSP, and CRYM to a final R = 0.199. Compound 6 was found to crystallize in space group $P2_12_12_1$ with cell dimensions a = 12.064(5), b = 16.222(4), c = 11.723Å, with Z = 4.

Similar methods, using crystals mounted in Seward and mailed to Akron, were employed to solve the structure of 12.

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